Crossbreeding of natural *Saccharomyces cerevisiae* strains for enhanced bio-ethanol production

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Summary

The fluctuating fuel prices, possible future shortage of fossil fuels, the increasing demand and the negative impacts on the ecosystem have all contributed to the search and development of alternatives during the last two decades. Biofuels, bio-ethanol in particular, is a reliable substitute for fossil fuel (petroleum) and can be produced from inexpensive, non-edible feedstock such as lignocellulosic biomass. Lignocellulose is, however, a problematic substrate as the hydrolysis results in inhibitor formation that hinders the fermentation ability of the fermenting microorganism (*Saccharomyces cerevisiae* yeast strain). The problem can be circumvented by the construction of robust *S. cerevisiae* strains that can withstand the effect of inhibitors in addition to exhibiting fermentation vigour, ethanol tolerance, inhibitor tolerance, osmotolerance and thermotolerance.

In this study, four natural strains of *S. cerevisiae* (HR14, YI64, YI2 and MF15) with different superior characteristics (fermentation vigour, inhibitor-, osmo-, thermo- and ethanol tolerance) were selected for mating experiments to generate hybrid progeny with superior traits. The *HO* gene of the diploid homothallic yeast strains was disrupted to produce haploid heterothallic strains. Haploid strains with the opposite mating-types and displaying different characteristics were mated to produce hybrid strains with combined / superior characteristics. Six hybrid strains (YH1, YH2, YH3, MY3, MY5 and MY7) were selected for the screening process.

The parent and hybrid strains were screened for fermentation vigour, ethanol tolerance, inhibitor tolerance, growth at temperatures above 30°C and osmotolerance. The YH3 and MY5 hybrid strains displayed the highest fermentation vigour (productivity) of the hybrid strains and were able to consume all available glucose (200 g/L) and produce approximately 100 g/L and 81 g/L ethanol, respectively. These hybrid strains did however, not display superior fermentation abilities when compared to the parent YI64 and YI2 strains as these strains produced the same amount of ethanol during the fermentation trials. The MY5 hybrid exhibited an inhibitor tolerance, similar to the MF15 parental strain in the presence of 25% inhibitor cocktail. The HR14 and YI64 parental strains and their YH1, YH2 and YH3 hybrid strains were unable to grow and ferment in the presence of 25% inhibitor cocktail. None of the strains was able to grow and ferment in the presence of 10% ethanol. Some inherited characteristics (fermentation vigour and inhibitor tolerance) of the hybrid strains were not superior to that displayed by the parental strains. The inherited osmotolerance and

thermotolerance were, however, superior to that displayed by the parent strains as the best performing hybrids managed to grow at 43°C and grew slightly faster than the parent strains in the presence of 65% glucose. The mating experiments yielded hybrid strains with combined characteristics such as fermentation vigour, inhibitor tolerance, osmotolerance and thermotolerance.

Mating of yeast strains to combine and generate superior traits in the progeny is thought to be the best method to use. Hybrid strains generated during this method are produced through minimum gene manipulation. The use of these strains in the production of bio-ethanol should not cause public concerns nor should it infringe on legislation. The mating experiments can be followed by an adaptation to inhibitory compounds, as the hybrid strains in this study were slightly more tolerant to ethanol during the fermentation trials after adaptation.

Opsomming

Die wisselende brandstofpryse, moontlike toekomstige tekort van fossielbrandstowwe, die toenemende aanvraag en die negatiewe impak op die ekosisteem het alles bygedra tot die soek en die ontwikkeling van alternatiewe gedurende die laaste twee dekades. Biobrandstof, bio-etanol in besonder, is 'n betroubare plaasvervanger vir fossielbrandstof (petroleum) en kan geproduseer word van goedkoop, nie-eetbare roumateriaal soos lignosellulose biomassa. Lignosellulose is egter 'n problematiese substraat, want die hidrolise daarvan lei tot die vorming van inhibitore wat die fermentasievermoë van die vergistende mikroörganisme (*Saccharomyces cerevisiae* gisras) verhinder. Die probleem kan egter omseil word deur die konstruksie van robuuste *S. cerevisiae* gisrasse wat die effek van inhibitore kan weerstaan bykomend tot die toon van fermentasiekrag, etanolverdraagsaamheid, inhibitorverdraagsaamheid, osmotoleransie en termotoleransie.

In hierdie studie is vier natuurlike stamme van *S. cerevisiae* (HR14, YI64, YI2 en MF15) met verskillende voortreflike eienskappe (fermentasiekrag, inhibitor-, osmo-, termo- en etanol verdraagsaamheid) gekies vir parings eksperimente om ŉ hibried nageslag te genereer met verbeterde eienskappe. Die *HO*-geen van die diploïede homotalliese gisstamme was ontwrig om haploïede heterotalliese stamme te produseer. Haploïede stamme met die teenoorgestelde paring-tipes wat verskillende eienskappe getoon het, was gekruis om hibried stamme met gekombineerde / verbeterde eienskappe te produseer. Ses hibried stamme (YH1, YH2, YH3, MY3, MY5 en MY7) was gekies vir die keuringsproses. Die ouers en hibried stamme was gekeur vir hul fermentasiekrag, etanolverdraagsaamheid, inhibitorverdraagsaamheid, groei by temperature bo 30°C en osmotoleransie. Die YH3 en MY5 hibried stamme het die hoogste fermentasiekrag (produktiwiteit) van al die hibried stamme vertoon en was in staat om alle beskikbare glucose (200 g/L) te verbruik en het ongeveer 100 g/L en 81 g/L etanol, onderskeidelik geproduseer. Hierdie hibried stamme het egter nie beter fermentasie vermoëns in vergelyking met die ouers YI64 en YI2 vertoon nie, want die ouers het dieselfde hoeveelheid etanol tydens die fermentasie proewe geproduseer. Die hibried MY5 het 'n inhibitor verdraagsaamheid, soortgelyk aan die MF15 ouerstam in die teenwoordigheid van 25% inhibitor mengsel getoon. Die HR14 en YI64 ouer stamme en hul YH1, YH2 en YH3 hibried stamme was nie in staat om te groei en te fermenteer in die teenwoordigheid van 25% inhibitor mengsel nie. Nie een van die stamme was in staat om te groei en te fermenteer in die teenwoordigheid van 10% etanol nie. Sommige oorgeërfde eienskappe (fermentasiekrag en

inhibitorverdraagsaamheid) van die hibried stamme was nie beter as wat vertoon was deur die ouer stamme nie. Die oorgeërfde osmotoleransie en termotoleransie was egter beter as wat vertoon was deur die ouer stamme, want die bes presterende hibriede het daarin geslaag om te groei by 43°C en het effens vinniger as die ouer stamme in die teenwoordigheid van 65% glukose gegroei. Die parings eksperimente het dus hibried stamme opgelewer met gekombineerde eienskappe soos fermentasiekrag, inhibitorverdraagsaamheid, osmotoleransie en termotoleransie.

Paring van gisstamme om verbeterde eienskappe in die nageslag te kombineer en te genereer is van mening dat die beste metode om te gebruik. Hibried stamme wat tydens hierdie metode gegenereer word bevat minimum geen manipulasie. Die gebruik van hierdie stamme in die produksie van bio-etanol hoort nie openbare kommer veroorsaak of teen wetgewing gaan nie. Die paringeksperimente kan gevolg word deur 'n aanpassing teen inhiberende verbindings, want die hibried stamme in hierdie studie was effens meer verdraagsaam teen etanol tydens die fermentasie proewe na die aanpassing.

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Table of Content

List of Abbreviations

1G:	First generation
2G:	Second generation
ARC:	Agriculture Research Council
bp:	Base pairs
CBP:	Consolidated Bioprocessing
CO_2:	Carbon dioxide
ddH_2O:	Double Distilled Water
DIG:	Digoxigenin
DNA:	Deoxyribonucleic Acid
DRR:	DNA Recognition Region
EIA:	Energy Information Administration
EMS:	Ethyl Methane Sulfonate
FF:	Furfural
FFV:	Flex-fuel Vehicles
G1:	Growth phase
gDNA:	Genomic DNA
GMO:	Genetically Modified Organism
GRAS:	General Regarded As Safe
HINT:	Hedgehog and Intein
HMF:	Hydroxymethylfurfural
HO:	Homothallism
ho:	Heterothallism
HPLC:	High Performance Liquid Chromatography
LSD:	Least Significant Difference
M:	Meiotic phase
MAT:	Mating-type-determining
MIC:	Minimum Inhibition Concentration
MNNG:	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
PJ:	Petajoule
PCR:	Polymerase Chain Reaction
RNA:	Ribonucleic Acid
S1:	Synthesis phase
SAS:	Statistical Analysis System
SGD:	*Saccharomyces* Genomic Database
UV:	Ultraviolet
WEC:	World Energy Council
WEO:	World Energy Outlook

Chapter 1: Introduction

1.1 Background

The shortage of fossil fuels, the increasing fuel demand and the rise of petroleum-based fuel prices, have become global concerns (Balat, 2011; Gasparatos *et al*., 2011; Gray *et al*., 2006; Nigam & Singh, 2011; Parisutham *et al*., 2014; Subhadra & Edwards, 2010; Yamada *et al*., 2010). The consumption of fossil fuels has a negative impact on the environment due to its greenhouse gas effect. Gas emissions produced by the transport sector contributes approximately 19% of the world's carbon dioxide (CO_2) production (Balat, 2011). Other environmental concerns include climate change, the retracting of the glaciers that result in a rise in the sea level and the loss of biodiversity (Dias *et al.*, 2009; Gasparatos *et al*., 2011; Hill *et al*., 2006; Nigam & Singh, 2011). Despite these concerns, fossil fuels still account for 80% of the primary energy consumed globally, with the transport sector using approximately 58% of the fossil fuels (Balat, 2011; Dias *et al.*, 2009; Escobar *et al*., 2009; Nigam & Singh, 2011).

Biofuels pose an attractive alternative to fossil fuels. It is environmentally friendly, requires inexpensive feedstock (plant biomass and derivatives thereof), is renewable (Macedo *et al*., 2008; Nigam & Singh, 2011), economically advantageous and biodegradable (Balat, 2011). One of the current challenges is that bio-ethanol is derived from agricultural feedstock such as sugarcane (sucrose) and corn (starch), so-called first generation (1G) biofuels. The critical and social concerns about the disturbance in global food supply and reduction in crop diversity (Subhadra & Edwards, 2010) shifted the focus to lignocellulosic materials, such as wood and waste materials (second generation biofuels, 2G) as an alternative feedstock (Fujitomi *et al*., 2012; Gasparatos *et al*., 2011; Hughes *et al*., 2009). It is clear that the increasing demand for an alternative fuel overshadows the negative perception associated with the production of bio-ethanol, as the need for alternative fuel sources is much greater.

The bio-ethanol production process involves the saccharification (chemical or enzymatic) of the biomass (carbohydrates derived from agricultural feedstock or waste products) into simple soluble sugars, followed by the fermentation of the sugars to ethanol by a microorganism (Argueso *et al.*, 2009). The ideal microorganism should be robust and able to ferment all available sugars present in the hydrolysate (Hughes *et al*., 2009). Several yeast

species have been identified as possible candidates for the conversion of biomass to ethanol, among which *Saccharomyces cerevisiae* is the most promising candidate.

The yeast *S. cerevisiae* has been used for decades in some of the largest and oldest biotechnology industries such as baking, brewing, distilling, winemaking and most recently, in the production of bio-ethanol (Bell *et al*., 1998; Benjaphokee *et al*., 2012; Tamai *et al*., 2000; van Zyl *et al*., 1993). As the model eukaryotic organism, *S. cerevisiae* is the ideal microorganism for genetic studies and the improvement of biotechnological processes (Bakalinsky & Snow, 1990). Methods such as genetic engineering, mutation selection, and protoplast-spheroplast fusion have facilitated the development of *S. cerevisiae* strains with novel genotypes (Bell *et al*., 1998; Tamai *et al*., 2000; van Zyl *et al*., 1993). The traditional mating technique, in which cellular fusion occurs, proved to be the most effective method for *S. cerevisiae* strain improvement (Bell *et al*., 1998). This technique has produced strains with improved performance, and thus more efficient and effective biotechnological processes.

Classical crossbreeding is a popular method used to combine haploid cells (of opposite mating-types) into new heterozygous diploid strains in a single procedure (Bizaj *et al*., 2012; Hashimoto *et al*., 2005; Pretorius, 2000; Romano *et al*., 1985). However, this method can be problematic, since most natural yeast strains are diploid, aneuploid or polyploid (Hashimoto *et al*., 2005; Tamai *et al*., 2000). These strains cannot be used to mate directly and haploid ascospores need to be obtained through sporulation (Hashimoto *et al*., 2005).

Another concern when using *S. cerevisiae* strains in mating experiments is the fact that these strains can either be homothallic or heterothallic. Homothallic strains are able to switch their mating-type thereby allowing self-mating. Heterothallic strains are unable to switch their mating-type and cannot self-mate (Tamai *et al*., 2000). The homothallic life cycle, which most natural strains display, is problematic because these strains are difficult to manipulate.

1.2 Research question

Can crossbreeding of natural *S. cerevisiae* yeast strains that display characteristics such as ethanol tolerance, inhibitor tolerance, osmotolerance, thermotolerance and fermentation vigour, through classical mating, enhance the production of bio-ethanol?

1.2.1 Null hypothesis

Crossbreeding of haploid *S. cerevisiae* strains with superior traits will deliver strains with enhanced / multiple characteristics for use in the production of bio-ethanol.

1.2.2 Alternative hypothesis

Crossbreeding of haploid *S. cerevisiae* strains with superior traits will not deliver strains with enhanced / multiple characteristics for use in the production of bio-ethanol.

1.3 Research aims

The primary aim of this study was to evaluate the application of classical crossbreeding in developing novel diploid *S. cerevisiae* strains with a robust nature to be used in a consolidated bioprocessing (CBP) process for the production of bio-ethanol on an industrial scale. The diploid strains were constructed by combining the characteristics of natural *S. cerevisiae* strains through mating. Since most natural strains are homothallic, it was necessary to convert these strains to heterothallism through the inactivation of the *HO* gene prior to mating.

The specific aims of this study included the following:

- To create heterothallic strains from natural homothallic *S. cerevisiae* strains, thereby generating haploid strains with desired traits such as high ethanol tolerance, fermentation vigour, tolerance to inhibitors, osmotolerance and the ability to grow at high temperatures.
- To mate the selected haploid *S. cerevisiae* strains to produce hybrids with multiple / improved characteristics.

1.4 Research objectives

To attain the research aims, the following objectives were identified:

- Select diploid natural *S. cerevisiae* strains that were able to ferment glucose effectively, whilst also being ethanol tolerant, inhibitor tolerant, osmotolerant and thermotolerant.

- Inactivate the *HO* gene responsible for switching of the mating-type in selected diploid strains through gene disruption and isolate the haploid progeny.
- Mate stable haploid strains (of opposite mating-types) that display different characteristics.
- Evaluate the diploid hybrid *S. cerevisiae* strains for glucose fermentation, ethanol tolerance, inhibitor tolerance, osmotolerance and thermotolerance.

1.5 Significance of the research

Through crossbreeding of two strains with different characteristics, the hybrid progeny could have combined / enhanced superior characteristics. These characteristics could lead to the improvement of the production of bio-ethanol in terms of having a robust strain that is able to withstand the harsh conditions commonly found in the production of bio-ethanol. The conditions that are common to this process is fermentation temperatures above 30°C, ethanol concentrations above 10% and inhibitors typically found in the lignocellulosic hydrolysates.

Chapter 2: Literature Review

2.1 Energy crisis

Modern economies are powered by fossil fuels due to its use in various industries, the transportation sector and the generation of electricity. Fossil fuels are formed from the fossilized remains of plant and animal matter that are exposed for millions of years to pressure and heat in the earth's crust. The significant increase in the use of fossil fuels due to urban development has led to it becoming the primary source of energy. The most commonly used fossil fuels are petroleum, coal and natural gas (Pimentel & Patzek, 2006). The United States of America (USA) consumes approximately 20 million barrels of crude oil per day (Azadi *et al*., 2012; Gray *et al*., 2006). Due to the high demand of fossil fuel, there has been a strain on the oil supply as oil consumption exceeds production, which has led to rapid fuel price increases (Atilgan & Azapagic, 2014). It has been established that the fuel reserves are dependent on two factors, namely the consumption rate and selling price (Shafiee & Topal, 2010). These two factors are interlinked; as the price drops, the consumption increases and *vice versa*.

In 2006, nine worldwide locations with remaining fossil fuel reserves were identified by the World Energy Council (WEC) (Shafiee & Topal, 2010). These reserves are expected to diminish by 2030 as predicted by the World Energy Outlook (WEO) in 2007 (Shafiee & Topal, 2009) therefore, other energy sources still need to be found. Another reason for the unsustainability of fossil fuels is that they are non-renewable sources of energy. In 2007, the Energy Information Administration (EIA) also projected that the global energy utilisation will increase with an average of 1.1% annually, but the energy consumption already reached an estimated 2% in 2009 (Shafiee & Topal, 2010). The trend of intensive use of fossil fuels has been set by humanity and other arrangements need to be made regarding alternative sources of energy as the reserves might be depleted before the predicted deadline.

The use of fossil fuels has an environmental impact as the combustion of these fuels leads to greenhouse gas emissions and global warming (Azadi *et al*., 2012; Escobar *et al*., 2009; Gray *et al*., 2006). The biggest source of greenhouse gas emissions is CO_2. The emission of this gas contributes significantly to the changes in the atmosphere. The atmospheric concentration of CO_2 has reached its highest level over the past century due to the intensive use of fossil fuels (Escobar *et al*., 2009).

The combustion of fossil fuels has also led to major changes in the global ecosystems (Escobar *et al*., 2009). These changes include rising in the acid levels of the oceans, an increased extinction risk of the earth's species, reduction in crop productivity, increased droughts and rise of the sea level (Suranovic, 2013). There has also been a steady rise in the earth's average temperatures, which led to problems such as bushveld fires, floods, water shortages, hunger, increase risk of skin cancer and deaths due to diseases like malaria (Escobar *et al*., 2009). According to a projection done by Wang *et al*. (2007), CO_2 emissions could be reduced by 60 to 90% if fossil fuels are replaced by biofuels (Kricka *et al*., 2015). To reduce the CO_2 release by the transport sector, a new approach therefore has been launched to produce fuel that has minimal to no harmful effects to the environment and that is renewable at the same time (Hasunuma *et al*., 2013; Hasunuma & Kondo, 2012). The focus has therefore shifted to biofuels as alternative fuels.

Biofuels are a winning substitute for fossil fuels. Biofuels are fuels that are derived from biological sources and can be classified as primary and secondary biofuels (Azadi *et al*., 2012). Primary biofuels are used for heating, cooking and electricity production, whereas secondary biofuels are used for natural processes and as transportation fuel (Nigam & Singh, 2011). Biofuels can further be divided into several categories, which include bio-ethanol, bio-diesel, bio-methanol and bio-hydrogen (Demirbas, 2007; Nigam & Singh, 2011). Bio-ethanol is a reliable substitute for and can be blended with petroleum. Bio-ethanol is currently the most promising alternative to the conventional fossil fuels (Hasunuma *et al*., 2013; Hasunuma & Kondo, 2012).

2.2 Bio-ethanol

Bio-ethanol is currently used on a large scale as a supplement for fossil fuels (Margeot *et al*., 2009) and offers a number of desirable features. Apart from limiting the greenhouse gas emissions, bio-ethanol has a secure and sustainable source of supply, has limited divergence with land-use for food and feed production, limits the fossil fuel input and contributes to a cleaner environment (Fujitomi *et al*., 2012; Margeot *et al*., 2009). It also displays characteristics such as a high octane value and good combustion efficiency making it ideal for use as a transport fuel (Hasunuma & Kondo, 2012).

Europe, China and African countries such as Kenya and Zimbabwe have made use of biofuels as a transportation fuel (Timilsina & Shresta, 2011). The two countries that make

sufficient use of bio-ethanol production are the USA and Brazil (Azadi *et al*., 2012). The amount of bio-ethanol produced jointly by Brazil and the USA amounts to 87% of the world's bio-ethanol production (Martínez *et al*., 2013). Currently, the USA is the largest producer of bio-ethanol, mainly focusing on the production of bio-ethanol from corn starch (Azadi *et al*., 2012; Gray *et al*., 2006). The USA has the capacity to produce up to 13 billion gallons of bio-ethanol per year from starch alone. Their goal is to produce up to 7.5 billion gallons of bio-ethanol per year (Gray *et al*., 2006). Recent trends have shown that the production of bio-ethanol have spiked, especially in the USA. A total of 75 billion litres are being produced annually of which the USA produces 50 billion litres and the rest is being produced by Brazil (Demirbas, 2007; Nielsen *et al*., 2013).

Brazil launched their ethanol program in 1979 when the oil prices climaxed (Timilsina & Shresta, 2011) by using sugarcane syrup as a substrate for bio-ethanol production, whereas the European countries use wheat, barley, grapes and sugar beet as feedstock (Kasavi *et al*., 2012). The production of bio-ethanol in Brazil has increased significantly over the past decade (Martínez *et al*., 2013; Walter *et al*., 2011). In 2011, the production of bio-ethanol reached over 1780 petajoule (PJ), amounting to about a third of the world's bio-ethanol production (Martínez *et al*., 2013). The majority of bio-ethanol produced by Brazil is used locally and less than 20% is exported to other countries (Azadi *et al*., 2012; Martínez *et al*., 2013). Brazil produces bio-ethanol at the lowest production cost possible and their bio-ethanol production program is not subsidized by the government (Walter *et al*., 2011).

In Brazil, cars with gasoline engines use a bio-ethanol-petroleum blend containing 24% bio-ethanol. Cars with flexible fuel engines can use any blend (Macedo *et al*., 2008; Walter *et al*., 2011). The introduction of flex-fuel vehicles (FFV) in 2003 spiked the utilisation of blended bio-ethanol fuel because of the higher ability to respond to price changes (Walter *et al*., 2011). Alcohol fuelled passenger vehicles can operate on 100% ethanol (Macedo *et al*., 2008). However, the recent trend in Brazil is to sell only FFVs rather than neat-ethanol cars (Walter *et al*., 2011).

The disadvantage to bio-ethanol use is that its production is hindered by several factors. The major factors include the recalcitrant nature of the biomass used and the high cost of the enzymes (cellulases and hemicellulases) needed to convert the biomass to sugar for the production of bio-ethanol. However, the advantages of bio-ethanol is that it burns much cleaner than gasoline, which makes it the favoured transport fuel above gasoline (Hoon &

Hyun, 2014). It can also be used as part of a blend and thereby reduce the net emission of greenhouse gases in the atmosphere, thus resulting in an overall decrease in ozone formation (Escobar *et al*., 2009). Bio-ethanol is produced from renewable energy resources and less expensive feedstock that would normally accumulate as waste products in the environment. Bio-ethanol is therefore environmentally friendly and biodegradable. Bio-ethanol production could also promote job creation, especially in the less developed countries (Coelho, 2005). For these reasons, considerable attention is drawn towards the production of bio-ethanol as this fuel has many advantages above the use of gasoline.

2.2.1 Bio-ethanol production

Bio-ethanol is the end product during the alcoholic fermentation of simple sugars, as depicted in Figure 1 (Demirbas, 2007). This is a two-step process where the first step is the saccharification of the biomass (cellulose, hemicellulose, sugarcane bagasse and starch) into simple sugars by enzymes or chemicals. The second step involves the fermentation of the sugars into ethanol, usually performed by a single yeast strain or a consortium of yeast strains.

The production of first generation (1G) bio-ethanol has a negative effect on third-world countries that rely heavily on agricultural land as a source of food security. The diversion of feedstock from the food supply chain to bio-ethanol has therefore raised much concern, resulting in the search for other non-edible feedstock sources (Kasavi *et al*., 2012). Studies have shown that agricultural land is not the only source that can be utilised for the production of bio-ethanol. Cheap substrates such as lignocellulosic biomass from agricultural, industrial and municipal waste streams (sugarcane bagasse, agricultural waste, paper sludge, etc.), which normally accumulate in the environment and contribute to global warming, can be used as an alternative source for the production of bio-ethanol (Dias *et al*., 2009; Dwiarti *et al*., 2012; Lynd *et al*., 2005). This has led to the concept of a second generation (2G) bio-ethanol production industry. However, the energy input needed to produce bio-ethanol is very high and often out of reach for a developing country (Hill *et al*., 2006).

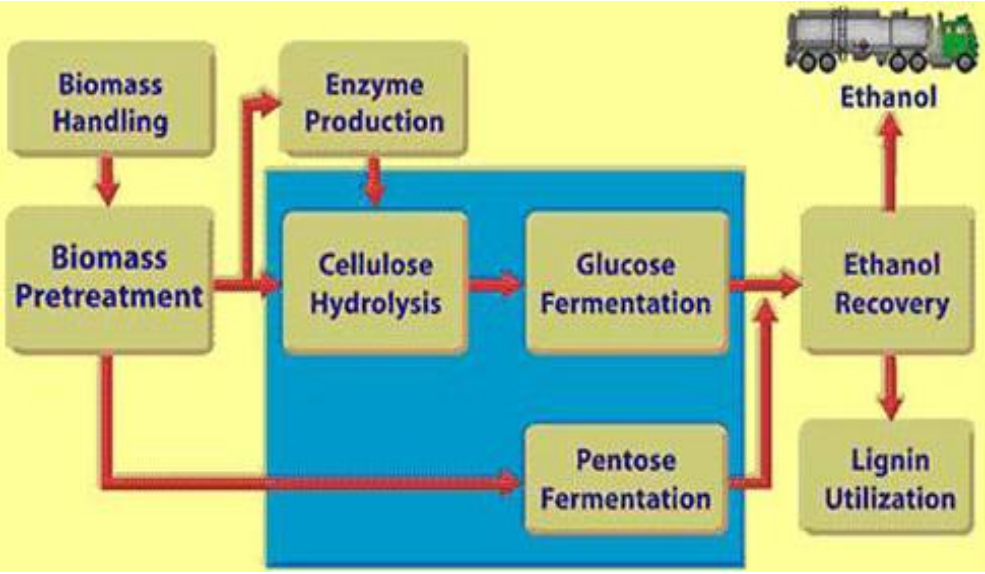

Figure 1: Steps in the production of bio-ethanol. Biomass is pre-treated to open up the crystalline structure of lignocelluloses. Next is the enzymatic hydrolyses of the cellulose and hemicellulose to simple sugars, followed by the fermentative conversion of the sugars to ethanol (www.power-technology.com).

The development of a 2G bio-ethanol production industry is delayed by economical and technical barriers (Margeot *et al*., 2009). Although the feedstock for 2G bio-ethanol production is cheaper than 1G bio-ethanol feedstock (Figure 2), the overall production cost is much higher (Cheng & Timilsina, 2011). Cellulases are also more expensive than the amylases used in the saccharification of starch.

The production of bio-ethanol from lignocellulose requires a pre-treatment step due to the recalcitrant nature of lignocellulosic biomass, which makes it resistant to enzymatic hydrolysis. Lignocellulose is by nature not a digestible material and has many chemical and physical barriers that resist enzymatic hydrolysis (Hoon & Hyun, 2014). Enzymatic hydrolysis can be inhibited by lignin, which protects the cellulose and hemicellulose fibers, as well as the acetyl groups of hemicellulose. The crystallinity, surface area, particle size, pore size and degree of polymerization of the lignocellulosic structures forms a physical barrier that inhibits the enzymatic hydrolysis of this substrate. Lignocellulose therefore needs to be pre-treated to render the cellulose and hemicellulose more accessible for hydrolysis; pre-treatment makes the process more expensive than the production of 1G bio-ethanol. Furthermore, most lignocellulosic materials have low densities that make them uneconomical

to pre-treatment (Cheng & Timilsina, 2011). The cost of these pre-treatment processes are increased by high chemical inputs and excessive water usage (Hoon & Hyun, 2014).

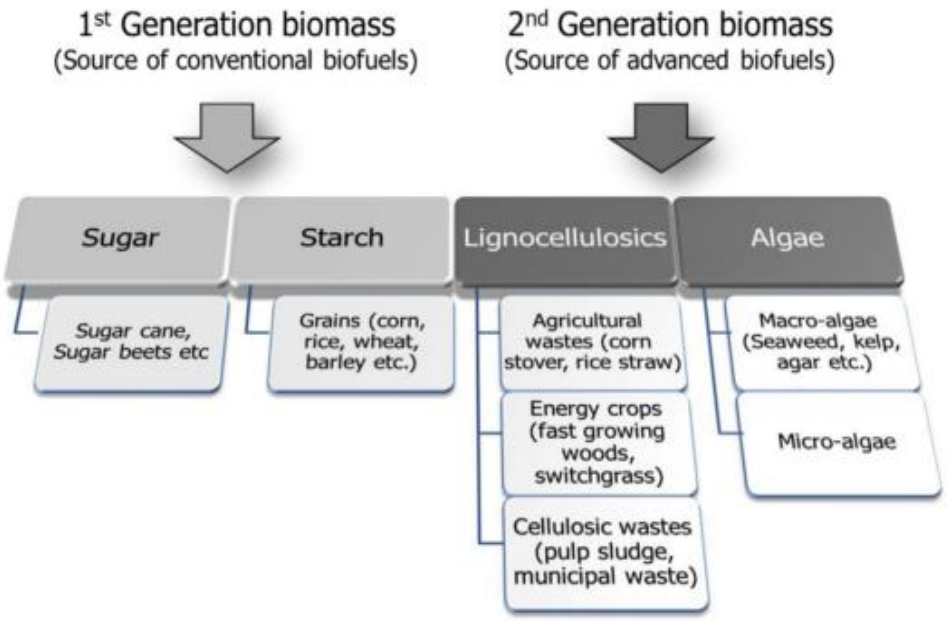

Figure 2: Classification of the different types of biomass (Hoon & Hyun, 2014).

Another obstacle that makes 2G bio-ethanol less economically feasible is the inefficient utilisation of the lignocellulosic materials. The current conversion of lignocellulose to bio-ethanol is in the range of 30 to 60%. The conversion rate can be much higher, but due to hemicellulose (30 to 85%) and lignin (0%) that have lower conversion rates than cellulose (85 to 90%), the overall conversion rate is lowered (Cheng & Timilsina, 2011). During enzymatic hydrolysis of hemicellulose, hexose and pentose sugars are produced. The latter cannot be fermented by yeasts, which leaves only the hexose sugars to be fermented. This significantly reduces the overall conversion rate. For this technology to be economically viable, certain areas need to improve such as the improvement of the pre-treatment processes and the application of less expensive commercial enzymes (Hoon & Hyun, 2014).

2.3 Feedstock resources

Plant biomass can be used as a feedstock for the production of bio-ethanol (Dias *et al*., 2009; Dwiarti *et al*., 2012; Kricka *et al*., 2015). Lignocellulose derived from plant cell walls is the most abundant organic compound found in nature (de Souza, 2013; den Haan *et al*., 2013;

Kim *et al*., 2013; Kricka *et al*., 2015). It consists of cellulose, hemicellulose, lignin and other components such as pectin, ash and extractives, as shown in Figure 3 (de Souza, 2013; Laluce *et al*., 2012). It is a renewable resource and has been used in the production of countless artificial products (Wong *et al*., 1988). Different types of biomass are currently being used for the production of biofuels. These include cellulose substrates such as sugarcane bagasse, wood biomass, agricultural waste, municipal solid waste, paper sludge and molasses. Starch accumulates in some plant cells and can also be converted to bio-ethanol; starch substrates include corn grain, potato, sweet sorghum and soybean (De Figueroa *et al*., 1984; Dwiarti *et al*., 2012; Gray *et al*., 2006; Subhadra & Edwards, 2010).

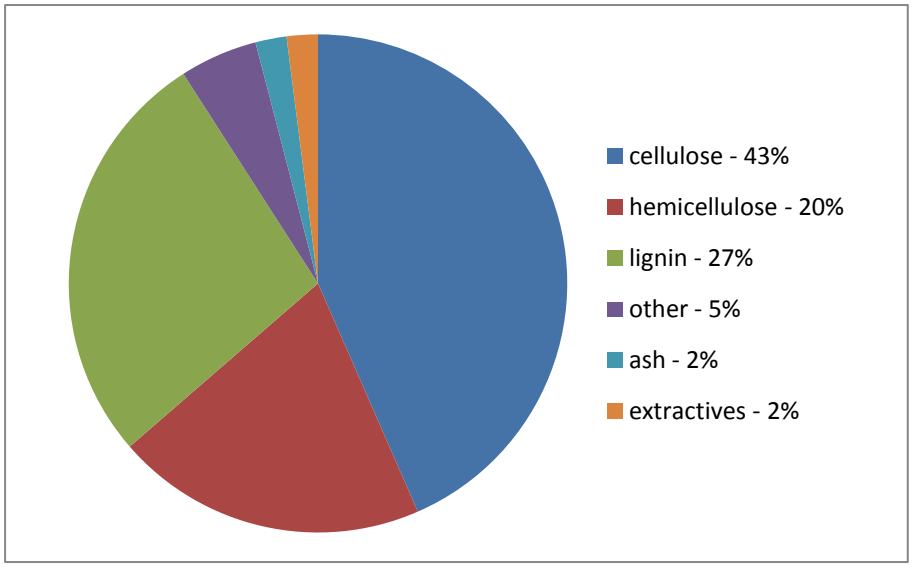

Figure 3: Typical composition of lignocellulosic biomass. Cellulose contributes to the largest portion of the lignocellulose, followed by hemicellulose and the other components (adapted from Dawson, 2011).

The hydrolysis of cellulose and hemicellulose is a critical step for the efficient conversion of lignocellulose to ethanol. However, due to the crystalline structure of cellulose, which is embedded in both hemicellulose and amorphous lignin, the enzymatic hydrolysis of cellulose is difficult (Hasunuma & Kondo, 2012). Pre-treatment of the lignocellulosic biomass is therefore important to break down the crystalline structure of cellulose and hemicellulose and to allow the ethanologenic microorganism to access the fermentable sugars.

Three main enzyme groups (endoglucanase, exoglucanase and β-glucosidase) are needed to degrade cellulose to fermentable sugars (Figure 4). These enzymes work in synergy to

achieve complete hydrolysis. Endoglucanase is required to break down the β-1,4-glycosidic bonds in the amorphous regions of the crystalline cellulose. Exoglucanase is responsible for the hydrolysis of the reducing and non-reducing ends of the cellulose chains, thereby releasing the cellobiose. β-Glucosidase converts the cellobiose into glucose (Kim *et al*., 2013; Yamada *et al*., 2010).

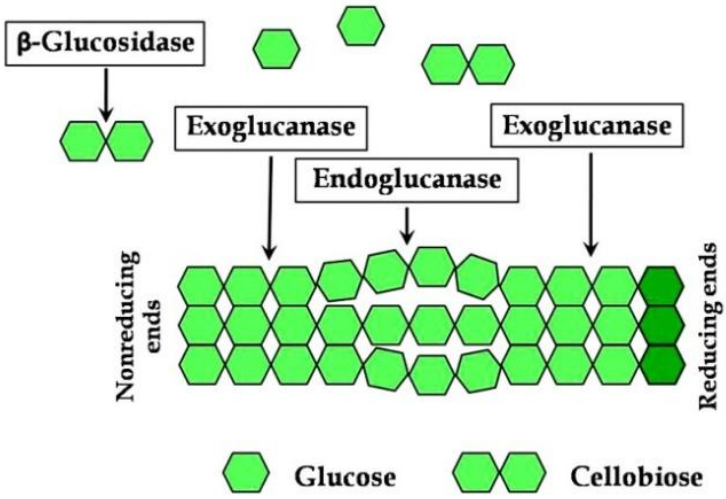

Figure 4: The enzyme complex needed for complete hydrolysis of cellulose (Ratanakhanokchai *et al*., 2013).

The enzymes needed for the hydrolysis of hemicellulose include endo-xylanase and β-xylosidase, along with the side chain splitting enzymes α-L-arabinofuranosidase, α-glucuronidase, acetyl and phenolic esterase (Figure 5). These enzymes degrade the xylan backbone to produce xylobiose and the final degradation of the xylobiose to xylose is achieved by the action of β-xylosidase (Hahn-Hägerdal *et al*., 2001).

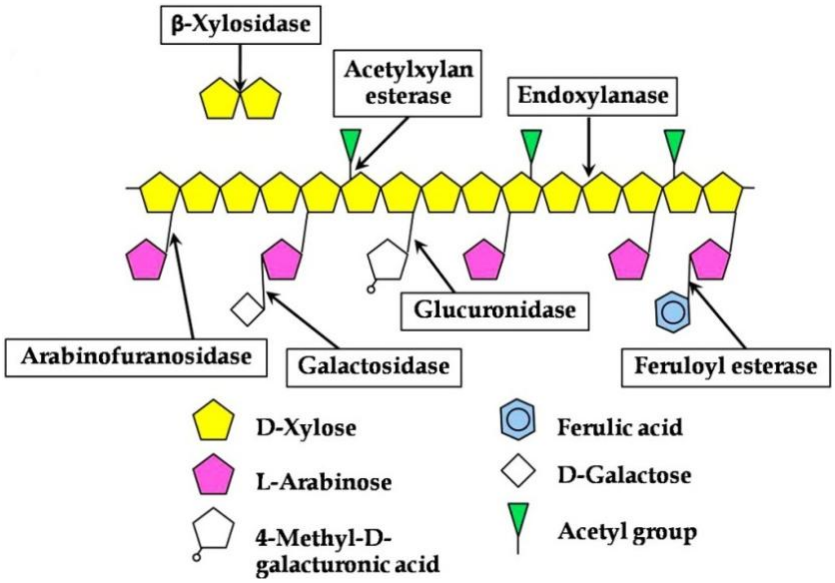

Figure 5: The major enzymes required to degrade hemicellulose to fermentable sugars (Ratanakhanokchai *et al*., 2013).

S. cerevisiae yeast strains are commonly used in the production of bio-ethanol. However, native *S. cerevisiae* strains are unable to hydrolyse the cellulose and hemicellulose into simple sugars and enzymes need to be added to the process (Hahn-Hägerdal *et al*., 2001). Commercial enzymes are expensive and increase the production cost of bio-ethanol.

During the past decades, a great deal of research has been conducted on the heterologous expression of cellulolytic and hemicellulolytic enzymes in *S. cerevisiae* (den Haan *et al*., 2015; Hahn-Hägerdal *et al*., 2001). Investigators have been working towards finding a way to omit the addition of commercial enzymes so that the bio-ethanol production process can be done in one step. This process is called consolidated bioprocessing (CBP), where enzyme production, saccharification and fermentation are done in a single step (den Haan *et al*., 2015; Hasunuma & Kondo, 2012).

2.4 Consolidated bioprocessing

The production of bio-ethanol from lignocellulosic materials usually involves the following steps: (1) a chemical or physiochemical pre-treatment step to release the cellulose and hemicelluloses; (2) hydrolysis of the cellulose and hemicellulose into fermentable sugars, usually done by enzymes or chemicals; (3) fermentation of the sugars into bio-ethanol,

normally achieved by a microbe or consortium of microbes and (4) concentration of the bio-ethanol by distillation and dehydration (de Souza, 2013; Hasunuma & Kondo, 2012).

The need for expensive enzymes to hydrolyse the polysaccharide chains into fermentable sugar units are eliminated within CBP, because the microorganisms produce their own cellulolytic and hemicellulolytic enzymes (Figure 6). The costs involved in CBP are therefore much lower as there are no additional costs related to enzymes (Lynd *et al*., 2005; Schuster & Chinn, 2012). CBP also eliminates the utilities associated with enzyme production, reduces the vessels needed for saccharification and fermentation, lowers the contamination risk and improves the hydrolysis process by mitigating product inhibition of cellulases and hemicellulases (Hasunuma & Kondo, 2012; Schuster & Chinn, 2012). CBP will significantly reduce the production cost of cellulosic ethanol, thereby reducing the overall capital investment and consequently increasing the use of ethanol as a biofuel (Kim *et al*., 2013).

CBP employs one of two strategies. In the first strategy, microorganisms that produce high yield and titer products (bio-ethanol), are engineered to express heterologous cellulase systems (den Haan *et al*., 2015; Olson *et al*., 2012). In the second strategy, microbes that have the ability to solubilise lignocellulosic biomass are engineered to produce high yield and titer products (bio-ethanol). However, the former strategy is widely accepted and seen as a more feasible option (Olson *et al*., 2012) with the potential to lower the cost of bio-ethanol production.

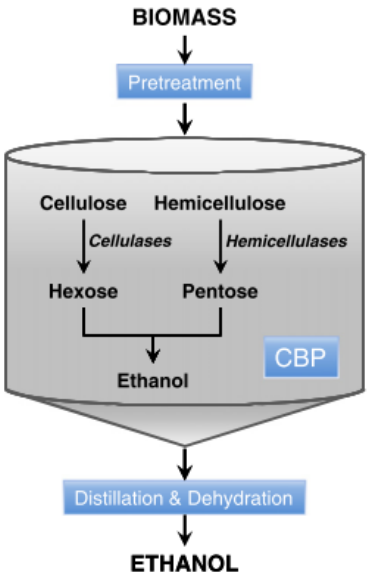

Figure 6: A schematic representation of the CBP process (Hasunuma & Kondo, 2012).

2.5 Microbial bio-ethanol production

A suitable microorganism for the commercial production of bio-ethanol should have the following characteristics: ability to produce high yields of bio-ethanol; (ii) ability to consume a broad range of substrates; (iii) tolerance to high temperatures; (iv) high ethanol tolerance; and (v) tolerance to the inhibitors present in the lignocellulosic biomass (Benjaphokee *et al*., 2012; de Souza, 2013). Several microorganisms have demonstrated the capability to degrade lignocellulosic biomass (Almeida *et al*., 2007; den Haan *et al*., 2013). Fungi and bacteria are the most commonly known cellulolytic microorganisms (de Souza, 2013). In the fungal group, *Trichoderma reesei* is the most important organism used in the production of cellulases, whereas *Aspergillus niger* produces a broad range of hemicellulases and has been used in many natural applications. Other fungi such as *Aspergillus kawachii*, *Aspergillus oryzae*, *Aspergillus aculeatus*, *Neurospora crassa* and *Pichia etchellsii* have also been reported to produce cellulases (Kim *et al*., 2013).

The cellulolytic bacteria include different genera such as *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Butyrivibrio*, *Acetivibrio*, *Cellulomonas*, *Erwinia*, *Escherichia*, *Zymomonas*, *Thermobifida*, *Fibrobacter*, *Cytophaga* and *Sporocytophaga* (Almeida *et al*., 2007; de Souza, 2013; Kim *et al*., 2013). The anaerobic bacteria species *Clostridium* and *Ruminococcus* are known to produce a cluster of enzymes (multi-enzyme machines, called cellulosomes), that facilitate efficient degradation of cellulose (de Souza, 2013; Kim *et al*., 2013). These microorganisms, however, do not meet all the requirements for a suitable bio-ethanol producer.

The yeast *S. cerevisiae* is the preferred organism for the production of ethanol as it is a facultative anaerobe that can ferment sugars efficiently under anaerobic conditions and has a robust nature that can tolerate high concentrations of ethanol (Benjaphokee *et al*., 2012; den Haan *et al*., 2013; den Haan *et al*., 2015; Fujitomi *et al*., 2012; Garay-Arroyo *et al*., 2004; Hahn-Hägerdal *et al*., 2001). Selected *S. cerevisiae* strains are also tolerant to low pH and handle osmotic pressure well (Hahn-Hägerdal *et al*., 2001; Le Borgne, 2012). These characteristics make them suitable candidates for genetic engineering. Several studies have been done to compare *S. cerevisiae*'s performance with other fermentative yeast and bacteria and found that *S. cerevisiae* outperforms all these strains (Hahn-Hägerdal *et al*., 2001). The drawback with *S. cerevisiae*, however, is that it does not have the capability to degrade lignocellulosic biomass as it lacks endogenous cellulases and hemicellulases. Another

concern is that *S. cerevisiae* does not have the ability to metabolise pentose sugars, although it can consume hexose sugars (den Haan *et al*., 2013; Lee *et al*., 2011). However, it is possible to engineer *S. cerevisiae* strains to hydrolyse lignocellulosic substrates and metabolise pentose sugars.

Strategy 1 (engineering *S. cerevisiae* to express heterologous cellulase systems) is more feasible as *S. cerevisiae* has the capability to produce high yields of bio-ethanol. Considerable improvement has also been made with regard to the expression of cellulases within this organism, which is easier than optimizing a strain to produce high concentrations of bio-ethanol.

Some success has been shown where *S. cerevisiae* was genetically engineered to convert cellulose and starch to ethanol. Den Haan *et al*. (2007) cloned a functional cellobiohydrolase in *S. cerevisiae*, enabling the yeast to convert cellulose to ethanol, despite low levels of expression. Van Rooyen and colleagues (2005) engineered a strain of *S. cerevisiae* to grow on cellobiose aerobically and anaerobically. Although these studies showed that expression of recombinant enzymes in *S. cerevisiae* strains still need to be optimised for efficient use, it supported the continued use of this organism as a CBP host.

The most probable *S. cerevisiae* strains to apply for CBP are natural strains as they are more robust by nature. Natural *S. cerevisiae* yeast strains differ significantly from laboratory strains as they have acquired superior traits from being exposed to a constantly changing environment (Garay-Arroyo *et al*., 2004). In contrast, laboratory yeasts are exposed to controlled environmental conditions. Laboratory strains have been modified over the years by classical genetic techniques and genetic manipulation such as meiotic tetrad analysis, genetic crosses of mutants, mutant isolation, etc. (Tamai *et al*., 2001). The traits that natural yeasts have acquired include (i) greater tolerance to fermentative by-products; (ii) substrate variability; (iii) tolerance to changes in the temperature; (iv) tolerance to high ethanol concentration; (v) tolerance to varying solute concentration; and (vi) tolerance to varying solute ionic strength (Garay-Arroyo *et al*., 2004). Natural yeasts are also more tolerant to the inhibitors (toxins) present in the hemicellulosic hydrolysate that arise from the pre-treatment of lignocellulosic biomass (Fujitomi *et al*., 2012; Garay-Arroyo *et al*., 2004).

Natural *S. cerevisiae* strains are often chosen for their ability to adapt to harsh environments. For example, they are able to survive the harsh fermentation conditions in the winemaking process, such as high temperatures and high ethanol content, whilst simultaneously

contributing to the quality of the wine through the production of secondary metabolites (Bizaj *et al*., 2012). These conditions are similar to the requirements for bio-ethanol production, except for a low pH and the presence of inhibitory compounds generated from the pre-treatment of the raw material or during the fermentation process itself (Pereira *et al*., 2012).

The manner in which laboratory strains have been genetically engineered are not applicable to natural strains due to the latter being diploid or polyploid (Tamai *et al*., 2001). Most natural strains are homothallic, whereas laboratory strains are heterothallic. The homothallic life cycle is exhibited by *S. cerevisiae* yeast strains that carry the functional homothallism (*HO*) gene, whereas the heterothallic life cycle is found in strains that carry the defective *ho* gene (Herskowitz, 1988). The *HO* gene encodes for an endonuclease that initiates mating-type switching in natural *S. cerevisiae* yeasts. Natural strains can therefore switch mating-types and spores of the same parent are able to mate. Laboratory yeasts are not able to switch mating-types and can therefore not mate with themselves (van Zyl *et al*., 1993).

2.6 Factors affecting bio-ethanol production

There are different factors that may affect the production of bio-ethanol. Lignocellulose is a renewable and cheap substrate that can be used for the production of bio-ethanol (Hasunuma & Kondo, 2012). In order for the fermenting microorganism to utilise the cellulose and hemicellulosic chains present in lignocelluloses, the lignocellulosic substrate needs to be pre-treated. The most common pre-treatment methods used are diluted acid hydrolysis and acid catalyzed steam explosion (Keating *et al*., 2006). Cellulose and hemicelluloses are released during this process, along with inhibitory compounds. These inhibitors have different mechanisms of affecting the production of bio-ethanol.

Inhibitors often affect the fermentation performance of strains. They may slow down the metabolism of the ethanologenic microorganism, thereby inhibiting growth or giving rise to a longer lag phase (Almeida *et al*., 2007; Almeida *et al*., 2009). Inhibitors may also have a cytotoxic effect on the microorganism through cytosol acidification, which could affect the cell's metabolic activity (Hasunuma & Kondo, 2012; Keating *et al*., 2005). These negative effects reduce the performance of the microorganism thus affecting the ethanol yield during fermentation.

It is therefore crucial to use a microorganism with tolerance to high ethanol concentrations, high inhibitor concentrations, high temperatures and high osmotic pressure. The most

common microorganism used in the production of bio-ethanol is *S. cerevisiae*, a robust organism that can withstand high concentrations of ethanol (Almeida *et al*., 2007). This organism is more tolerant to moderate concentrations of inhibitory compounds than bacteria and other fermentable yeasts (Almeida *et al*., 2007). *S. cerevisiae* also has a long standing relationship with different biotechnological industries and is generally regarded as safe (GRAS) (Lee *et al*., 2011). Though *S. cerevisiae* has the capability to tolerate high concentrations of ethanol, it cannot hydrolyse cellulose and hemicelluloses into fermentable sugars due to a lack of self-expressed cellulosic enzymes. This organism therefore needs to be engineered to breakdown a mixture of polysaccharides chains. It is also unable to tolerate high temperatures (45 to 50°C), which is a common condition during saccharification of cellulose in the bio-ethanol production process (La Grange *et al*., 2010; Lin *et al*., 2012).

2.6.1 Factors affecting the fermentation process

The yeast cells are subjected to a multitude of stresses that can have a negative effect on the production of bio-ethanol (Kumari & Pramanik, 2012). These stresses include high temperatures, high ethanol concentrations, toxic inhibitory compounds and high osmotic pressure.

2.6.1.1 Inhibitory compounds

Strains of *S. cerevisiae* have predominantly been used in the wine fermentation industry because of their natural association with grapes and the desirable characteristics displayed by these yeasts (Combina et al. 2012; Pérez-Través *et al*., 2012). However, one of the challenges faced by these strains on more complex substrates is the presence of inhibitory compounds in lignocellulosic hydrolysates. These inhibitors are released during the pre-treatment of biomass for the production of bio-ethanol (Fujitomi *et al*., 2012). The pre-treatment step is necessary to release the cellulose, hemicellulose and lignin fractions from the lignocellulose to make it accessible for hydrolysis (Laluce *et al*., 2012). The pre-treatment methods currently used include harsh conditions such as high pressure and temperatures, as well as the use of solvents that release various chemical compounds that have an inhibitory effect on the microorganism used for the fermentation process (Hou & Yao, 2012).

The pre-treatment method as well as the type of feedstock determines the type and concentration of inhibitory compounds released (Hou & Yao, 2012). The types of inhibitors

encountered can be divided into three categories namely furan derivatives, weak acids and aromatic compounds (Almeida *et al*., 2007; Almeida *et al*., 2009; Hou & Yao, 2012).

The dominant group of inhibitors in lignocellulosic hydrolysates is the furan derivative group (Almeida *et al*., 2009; Lee *et al*., 2011; Lin *et al*., 2009). This group of aldehydes consists of furfural (FF) and hydroxymethylfurfural (HMF), which are produced during the degradation of pentose and hexose sugars (Figure 7 & 8), respectively (Almeida *et al*., 2009; Hasunuma & Kondo, 2012; Lee *et al*., 2011). Although inhibitory during the production of bio-ethanol, these two compounds can be used in the production of fine chemicals and plastics (Almeida *et al*., 2009), or as a flavour compound in the case of FF.

Weak acids, such as levulinic acid and formic acid, are formed when HMF are further broken down during the pre-treatment process (Almeida *et al*., 2009). Acetic acid is formed when acetylxylan, part of the hemicellulose structure, is hydrolysed (Almeida *et al*., 2007; Lee *et al*., 2011). Formic acid is usually present at lower concentrations than acetic acid, but is more toxic to organisms such as *S. cerevisiae* (Hasunuma and Kondo, 2012). When lignin is degraded, aromatic compounds such as vanillin (Figure 8) and 4-hydroxybenzaldehyde are produced (Hasunuma & Kondo, 2012).

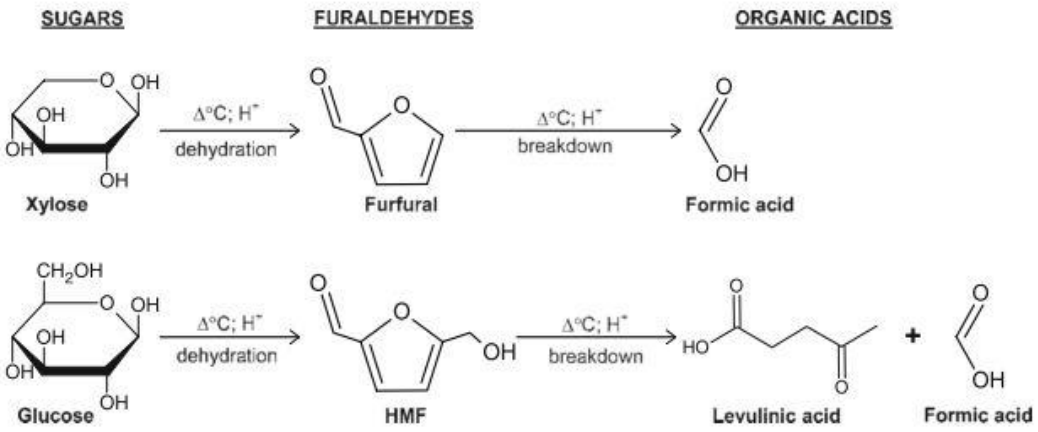

Figure 7: The production of various inhibitory compounds during the degradation of hexose and pentose sugars (Almeida *et al*., 2009).

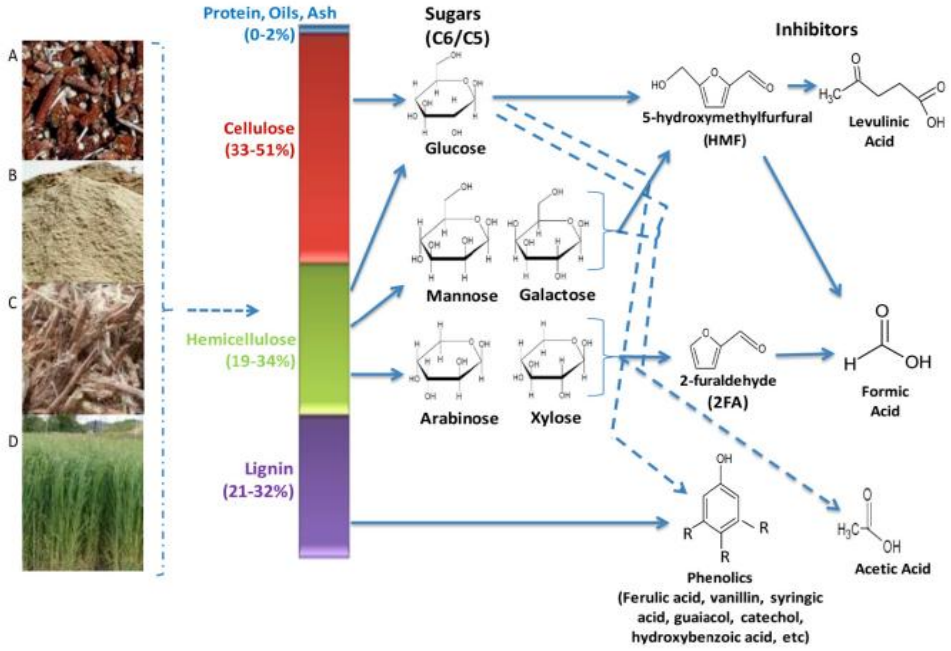

Figure 8: The inhibitory compounds generated from lignocellulose, in particular lignin (Ibraheem & Ndimba, 2013).

Effect of inhibitors on microbial metabolism

The inhibitory compounds affect cell growth as well as the metabolic activity of the organism during fermentation (Figure 9). In particular, FF has been known to cause DNA damage in eukaryotic cells, whereas HMF and acetic acid are known to have a cytotoxic effect on eukaryotic cells (Almeida *et al*., 2009; Keating *et al*., 2006). FF and HMF were also associated with a range of other negative effects that include cell wall damage, DNA breakdown, and inhibition of protein and RNA synthesis (Hasunuma & Kondo, 2012). Aromatic compounds act on biological membranes, causing loss of integrity and thus affect the ability of the membranes to serve as selective barriers and enzyme matrices (Hasunuma & Kondo, 2012).

Acetic acid at high concentration has various negative effects on cellular metabolism. In its undissociated form, it can move across the cell membrane and dissociate at physiological pH. This leads to acetate entrapment, intracellular proton build up and ultimately to acidification of the cytosol and postponement of metabolic processes (Keating *et al*., 2006). All of this takes place at the expense of ATP hydrolysis, thus less ATP is available for biomass formation (Almeida *et al*., 2007; Hasunuma & Kondo, 2012). Intracellular acidification also leads to a reduction in DNA and RNA synthesis. Acetic acid causes extensive degradation of

ribosomal RNA as a result of apoptotic mechanisms (Hasunuma & Kondo, 2012). Weak acids affect the cell's lipid organization and the function of the membrane embedded proteins.

Ethanol has various negative effects on the yeast cell, including growth and viability inhibition, inhibition of the glucose transport systems and promotion of proton movements across the cell membrane. It affects the plasma membrane with regard to its permeability, organization and lipid structure (Ansanay-Galeote *et al*., 2001; Jönsson *et al*., 2013). Ethanol disrupts the phospholipid bilayer of cell membranes, which causes leakage of the intracellular constituents and allows the entry of other substances that may be harmful or toxic to the cell. This may ultimately cause disturbance in the original composition of the cytoplasma, as the yeast does not have control over what enters or exits the cell, because there is no effective selective barrier (Quintas *et al*., 2000). As the homeostasis of the membrane is disturbed, intracellular acidification is promoted by a proton-motive force across the plasma membrane through the H^+-pump ATPase.

Reports have shown that an ethanol concentration of 4 to 6% (v/v) induces a stress response that causes the formation of heat shock proteins in yeast (Ansanay-Galeote *et al*., 2001). It has been found that with the increase in ethanol concentration, the pH of the living environment of the yeast decreases. This leads to the denaturation of the yeast enzymes as the bonds that holds them together starts to break or change (Quintas *et al*., 2000). High concentrations of ethanol are therefore detrimental to yeast as it may eventually lead to cell death.

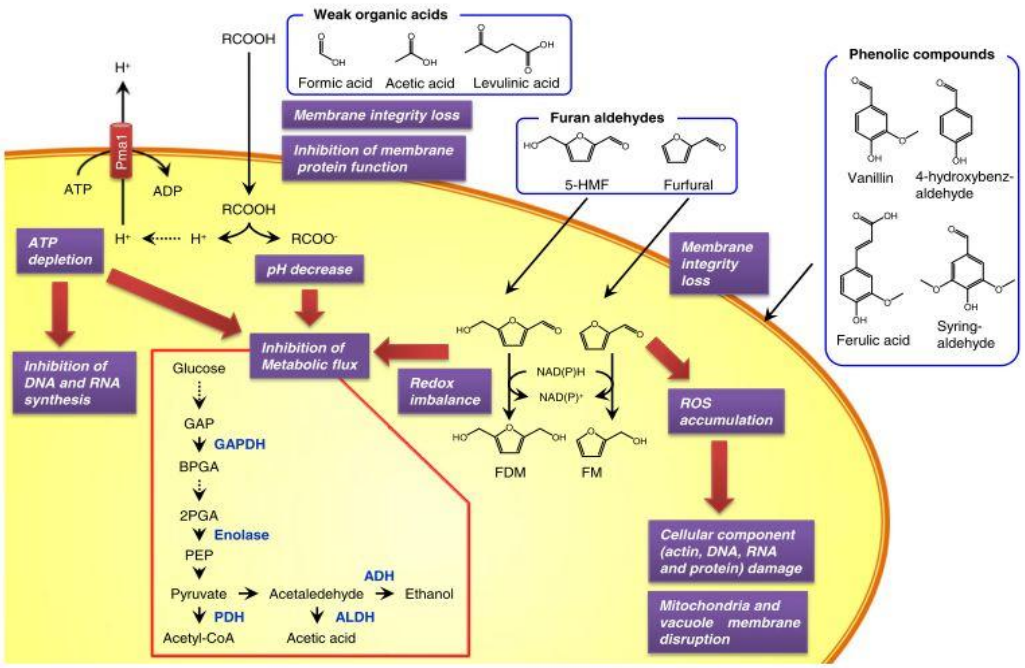

Figure 9: Metabolic effects of the inhibitory compounds on the cell (Hasunuma & Kondo, 2012).

Effect of inhibitors on microbial fermentation

Inhibitory compounds act through various mechanisms to reduce the efficiency of ethanol production (Fujitomi *et al*., 2012; Hou & Yao, 2012). FF and HMF cause the fermentative microorganism to enter an extended lag phase, thereby reducing the fermentation rate (Almeida *et al*., 2007; Almeida *et al*., 2009). The extended lag phase observed during fermentation can be due to the inhibition of various enzymes (Figure 10) including alcohol dehydrogenase (ADH); aldehyde dehydrogenase (AIDH); pyruvate dehydogenase (PDH); hexokinase (HK); and glyceraldehyde-3-phosphate dehydrogenase (G3PD) (Almeida *et al*., 2009). The extension of the lag phase has been said to be dependent on the microorganism used as well as the furan concentration (Almeida *et al*., 2007). Studies showed that furans directly inhibit glycolytic enzymes, because of a decreased activity observed (as measured *in vitro*) (Almeida *et al*., 2007). Furfural also causes vacuole and mitochondrial membrane damage as well as chromatin and actin damage in *S. cerevisiae* (Almeida *et al*., 2007; Hasunuma & Kondo, 2012).

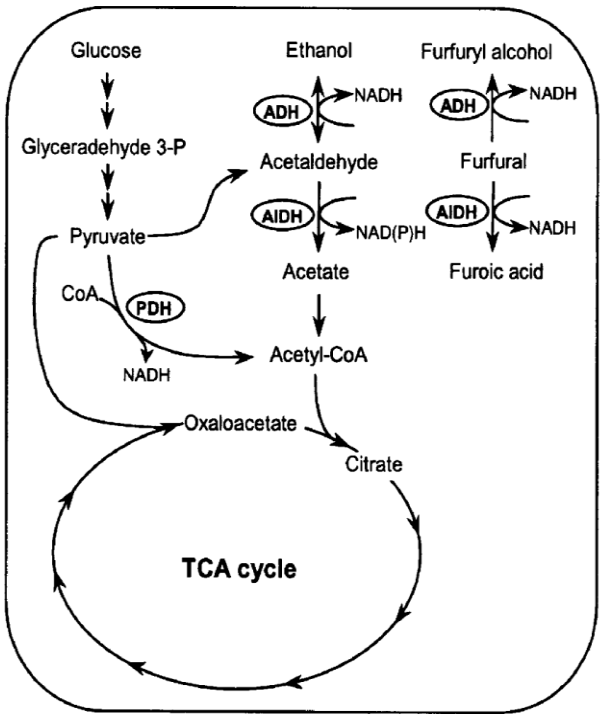

Figure 10: The interactions between the inhibitors and the glycolytic enzymes (Modig *et al*., 2002).

The fermentative organism should therefore be able to tolerate as well as ferment in the presence of these inhibitory compounds. The cell do this by decreasing the growth rate and converting the available furaldehydes to less volatile alcohol derivatives using an energy dependent reduction reaction during the lag phase (Figure 7 & 8) (Almeida *et al*., 2009; Keating *et al*., 2006). A study done by Lui *et al*. (2008) confirmed that multiple aldehyde reductases are involved in the conversion of furfural to their alcohol derivatives. The yeast re-directs its energy in fixing the damage caused by furans, which in turn can be observed by the reduced levels of ATP and NAD(P)H within the cell (Almeida *et al*., 2009). Growth after this detoxification action, can resume at a normal rate under anaerobic conditions (Keating *et al*., 2006).

2.7 *Saccharomyces cerevisiae* strain development

Natural strains of *S. cerevisiae* possessing a combination of the ideal characteristics required for the production of bio-ethanol are still being sought (Pérez-Través *et al*., 2012). Numerous techniques have been proposed for the improvement of such strains, but only a few could be applied to natural yeasts (Pérez-Través *et al*., 2012). An additional advantage for the use of *S. cerevisiae* as preferred organism is that the genome has been sequenced. The manipulation

of this organism's genome has been demonstrated, although most biotechnological techniques have been optimised for laboratory strains and might not be suitable for direct use in natural *S. cerevisiae* yeast strains. Mutagenesis, mating and genetic engineering are the most commonly used manipulating techniques.

2.7.1 Mutagenesis

The purpose of mutagenesis is to improve the genetic constitution of an organism (Kumari & Pramanik, 2012). There are various means of inducing mutations within a cell or a culture. Commonly used mutation strategies include random mutations and direct mutations. Random mutagenesis involves the use of DNA-damaging agents, whereas direct mutations involve genetic engineering or DNA manipulation.

2.7.1.1 Random mutagenesis

Random mutation is a standard procedure used in the study of genes and gene function. During random mutagenesis, DNA-damaging agents are used to create various lesions within the genetic material of a cell or cell population. The cell responds by repairing its DNA (Klein *et al*., 1989; Kumari & Pramanik, 2012). Unlike direct mutations, random mutations are not gene-specific, but focus on the cell as a whole. The chemical agents commonly used are ethyl methane sulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). These agents have been proven to be carcinogenic, mutagenic and recombinogenic (Klein *et al*., 1989). Treatment with these agents has also been found to affect different metabolic activities of the yeast strains. EMS has been found to alkylate DNA base pairs and leads to the transition of the adenine (A) to a thymine (T), and the guanine (G) to a cytosine (C), thereby causing point mutations (Kumari & Pramanik, 2012; Mobini-Dehkordi *et al*., 2008). MNNG on the other hand causes various lesions by reacting with the DNA. Ultraviolet (UV) light is also used as a DNA damaging agent; UV radiation leads to mitotic crossing over, mitotic gene switching and reverse mutation by the formation of cyclobutane dimers (Kumari & Pramanik, 2012).

2.7.1.2 Directed mutagenesis

This method specifically targets certain genes within the genome of a cell or cell population, thereby bringing about change within the genetic material. This can be achieved through polymerase chain reaction (PCR) based disruptions (Lorenz *et al*., 1995). Disruption cassettes

are constructed that will integrate within the genome of an organism (Figure 11) and recombine through homologous recombination of the endogenous gene with the homologous sequences from the disruption cassettes (Zaragoza, 2003).

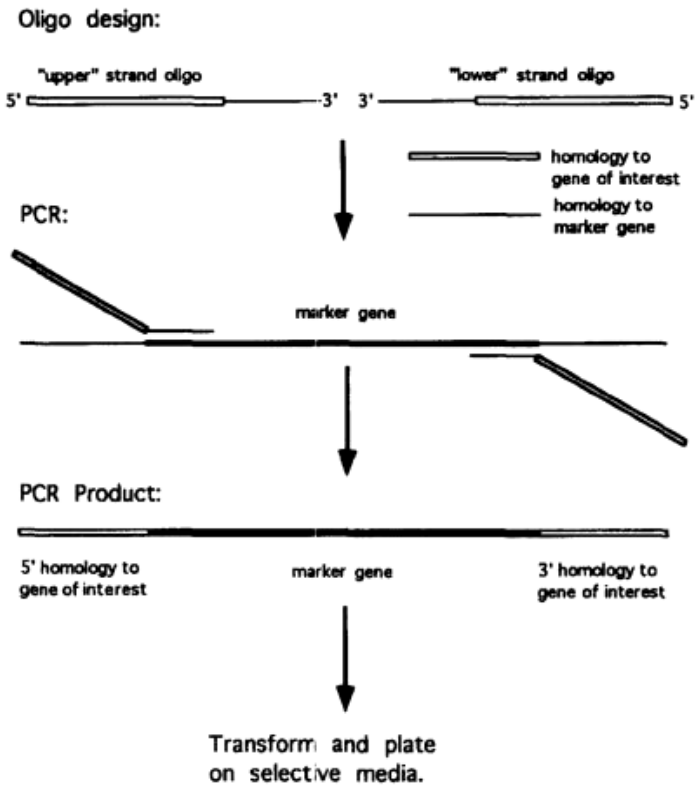

Figure 11: Illustration of a construction of a gene disruption cassette in *S. cerevisiae* (Lorenz *et al*., 1995).

For successful application of this technique, several steps are required. Firstly, the target gene to be disrupted needs to be identified. Secondly, the foreign DNA that will replace the target gene needs to specifically recombine into homologous regions of the genome (Lorenz *et al*., 1995). The foreign DNA is usually a selectable marker that will permit tracking of the integration event. In *S. cerevisiae*, dominant selectable markers can be used that confers resistance to a variety of antibiotics. A third requirement is a transformation system to introduce the DNA into the target cells (Lorenz *et al*., 1995). Lastly, a detection system needs to confirm successful integration and disruption of the target gene.

The first report of gene replacement was described by Scherer and Davis in 1979, where several hundred yeast genes were serially deleted and replaced. Gene replacement is generally used to understand the physiological processes that take place in an organism.

2.7.2 Mating

Hybrid strains can be obtained in three different ways, which include spore-to-spore mating, protoplast fusion and classical mating. Hybrids obtained through spore-to-spore fusion are thought to have weaker characteristics than their initial parental strains, because of the segregation of traits during the meiosis process prior to mating. Each hybrid therefore has a limited chance of inheriting all of the characteristics displayed by their parental strains. Hybrids obtained through protoplast fusion are considered as genetically modified organisms (GMO's) and due to public concerns and legislation in some countries, the use of GMO's are limited. The classical mating process, which involves the fusion of two complete genomes, are more accepted as the hybrids are not considered GMO's (Pérez-Través *et al*., 2012). Natural *S. cerevisiae* hybrid strains have previously been isolated from grapes and wine equipment; these strains have adapted to the harsh natural conditions. Having the characteristics of both parents make them ideal for application in processes such as winemaking, baking and in the production of bio-ethanol (Pérez-Través *et al*., 2012).

2.7.2.1 Classical mating

Strains of *S. cerevisiae* can exist as either haploid (either **a** or α) or diploid (**a**/α) variants. The mating-type locus of *S. cerevisiae* is located on chromosome III (Haber, 1998; Schiestl, 1989). Methods that are available for determining the mating-type includes mating the unknown mating-type strain with a strain with a known mating-type. Alternatively, the α-factor confrontation assay could be used, but the results can be ambiguous (Huxley *et al*., 1990). Analysing a strain's mating-type using PCR is a fast and reliable approach and was first described by Huxley *et al*. (1990).

Classical mating is set in motion through the ability of the strains to produce and respond to diffusible extracellular factors called pheromones (Herskowitz & Oshima, 1981; Hicks & Herskowitz, 1976; Jones & Bennett 2011; Merlini *et al*., 2013). Pheromones are chemical signalling molecules that trigger a social response in members of the same species. In yeast, these molecules activate a response of the opposite sex to mate. When an **a** and α yeast cell encounters mating pheromones in the opposite cell type, a cell surface receptor appears that allows the pheromone to bind to it. This action leads to the induction of genes necessary for mating and arrest the cell cycle in growth (G1) phase (Erdman *et al*., 1998; Hicks & Herskowitz, 1976; Merlini *et al*., 2013; Michaelis & Barrowman, 2012; Montelone, 2002).

Morphological changes occur where the cells undergo elongation into pear shapes, termed "schmooing" (Montelone, 2002). These alterations prepare the yeast cells for mating and fusion to form stable diploids. In order for the fusion process to take place, an intimate cell to cell interaction is necessary (Campbell, 1973). The pheromones are inactivated as soon as the cells agglutinate. The **a**/α diploids are therefore not responsive to mating pheromones of either type, but can be induced to undergo meiosis *via* nutrient deprivation and undergo sporulation (Herskowitz & Oshima, 1981). The mating process is illustrated in Figure 12.

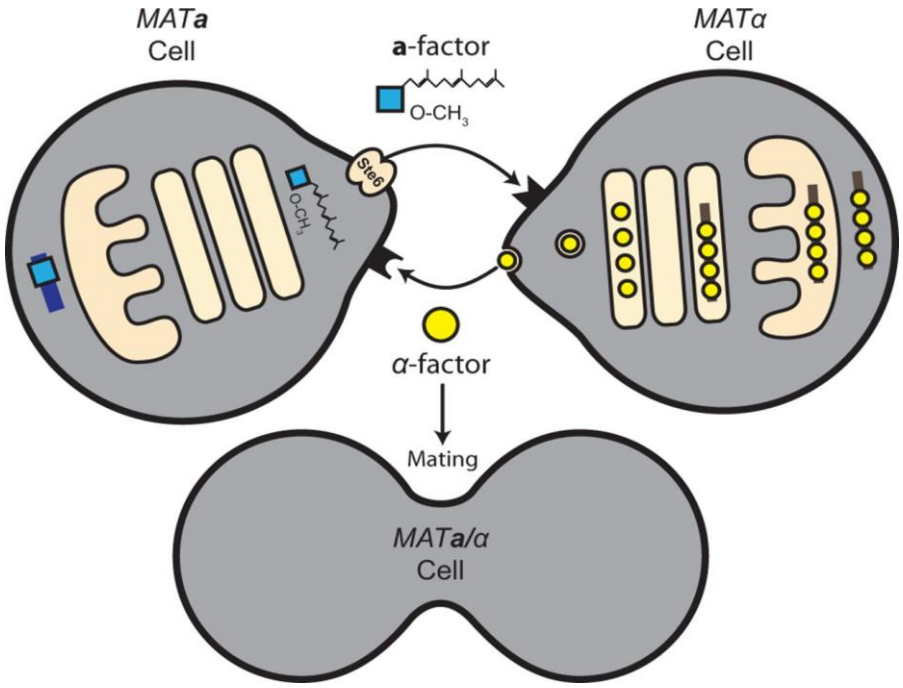

Figure 12: In the mating process, pheromones are signalling substances that pave the way for the mating of opposite cells. The *MAT***a** cell produces the a-factor, which attracts the *MAT*α cell and *vice versa* (Michaelis & Borrowman, 2012).

2.7.2.2 Protoplast fusion

Since the 1980's, the options available to natural microbiologist in terms of genetic manipulation of yeasts have expanded significantly (Scheinbach, 1983). Extensive progress has been made with recombinant DNA techniques where yeasts have been manipulated to express the human leukocyte interferon and new yeast strains have been developed through the use of protoplast fusion. During the last few decades, protoplasts were used as starting material for many natural applications as well as for basic research (Wöstemeyer & Wöstemeyer, 1998). Moreover, protoplast fusion has become a valuable tool to investigate

the molecular genetics of an organism or a species (Scheinbach, 1983). This method is frequently used to enhance the characteristics of microbial species where sexual mating is not possible (Loray *et al*., 1995; Wöstemeyer & Wöstemeyer, 1998).

Protoplast fusion is also seen as a way of constructing new yeast species for natural purposes (Janderová et al., 1986; Loray *et al*., 1995) and provides a means to analyse commercially valuable traits of certain species (Scheinbach, 1983). It is also commonly used to hybridize natural yeasts that are homothallic with a low sporulating ability (Nakazawa & Iwano, 2004; Tamai *et al*., 2001). Natural yeast are normally non-maters, because of their homothallic life cycle and polyploidy (Tamai *et al*., 2001).

The net charge on the protoplast cell surface is uniform and protoplasts therefore do not agglutinate spontaneously (Stahl, 1978). A number of events take place during protoplast fusion that is not evident during sexual mating (Klinner & Böttcher, 1985). During the fusion process, cybrids are formed (Figure 13), hybrids that contains the nucleus and cytoplasm of both parents (Janderová *et al*., 1986). This method is highly recommended for the improvement of natural yeasts. The strains participating in the hybridisation process should be transformed with a selective marker to allow easier selection of the fusion (hybridisation) product (Bell *et al*., 1998; Nakazawa *et al*., 1999; Nakazawa & Iwano, 2004). The dominant selective markers commonly used in this method are Tn*601(903)*, a Geneticin® resistance marker (antibacterial), and *AUR1-C*, an aureobasidin A resistance marker (antifungal). The advantage of protoplast fusion is the combination of two entire genomes, which increases the possibility of recombination, the transfer of cytoplasmic components and the rate of the transfer process (Gumpert, 1980). Hybrids formed by this method are, however, considered as GMO's according to the Directive 2001/18/EC of the European Parliament and the Council of the European Union (Pérez-Través *et al*., 2012). Their use in natural processes is therefore not accepted by many as there are conflicts with public concerns or legislation (Pérez-Través et al. 2012).

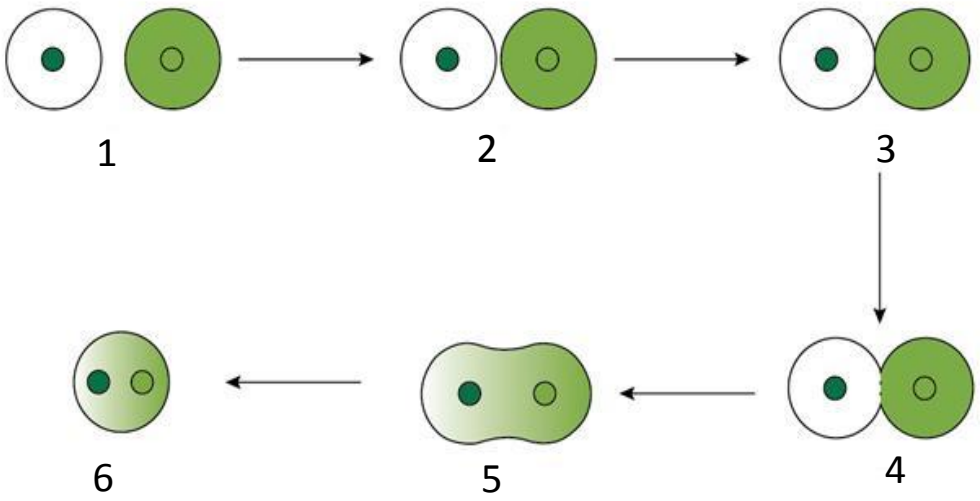

Figure 13: The different stages in protoplast fusion: (**1**) two separate protoplasts, (**2**) agglutination of the two protoplasts, (**3 and 4**) membrane fusion, and (**5 and 6**) formation of a heterokaryon (http://nptel.ac.in/courses/102103016/module1/lec13/5.html).

2.7.2.3 Mating of spore-derived clones

Another strategy used for the genetic improvement of yeast strains is mating of spore-derived clones, which has been extensively used in the wine industry (Nakazawa *et al*., 1999; Pérez-Través *et al*., 2012; Rainieri & Pretorius, 2000). This technique involves the fusion of two complete genomes. What makes this technique different from protoplast fusion is that sexual mating is an important requirement for the formation of the hybridisation products.

Natural yeast strains normally form diploid cultures by self-diploidization or intra-ascus mating, thus making mating by spore-derived clones a viable strategy (Murphy & Zeyl 2010; Nakazawa *et al*., 1999; Romano *et al*., 1985). Natural yeasts in general are diploid or polyploid and therefore cannot mate under normal conditions. Spores are formed upon sporulation, after which spontaneous mating can occur between germinating spores of opposite mating-types (Figure 14). This usually occurs in yeasts with a functional *HO* gene. A prerequisite for self-mating is to obtain the participating parental yeasts in a haploid phase, in other words in a stable ploidy state. After sporulation, the spores are separated by a micromanipulator and physically mated (Nakazawa *et al*., 1999).

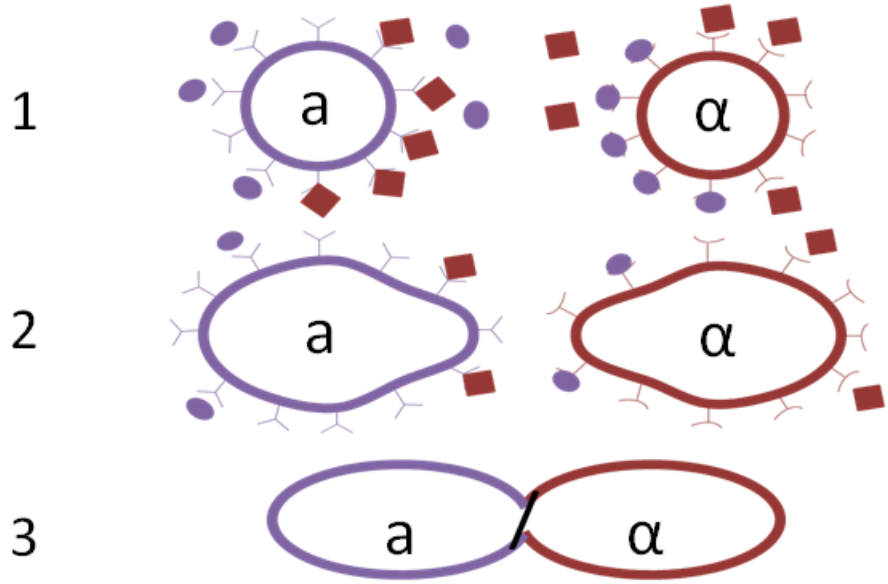

Figure 14: During the mating process of the spore-derived clones, pheromones are produced by the cells of the opposite mating types, which induce the mating process. Two haploid clones fuse to form **a**/α diploid (adapted from Fijalkowski, 2006).

Sporulation is a mechanism that the cell employs when the environmental conditions become unfavourable. The initiation of this event begins when the cell decides to differentiate into spores. This decision is based on a multitude of factors such as nutritional deficiency, high temperatures and the mating-type of the cell (Neiman, 2011). The cell adopts a different reproductive strategy to survive despite the harsh circumstances. The cell moves from the G1 phase of the cell cycle to the synthesis (S1) phase during early sporulation. Upon completion of this phase, the cell requires changes within the cell cycle machinery as well as alterations in RNA processing (Neiman 2011). The middle meiotic (M) phase includes meiotic divisions that give rise to four haploid nuclei packaged into daughter cells that are later surrounded by prospore membranes. In the late phase, the prospore membrane closes and thickens, after which each spore begins its maturation. The original mother cell disintegrates around the spore, to give rise to a tetrahedral mature ascus.

Ascospore formation is the result of sporulation during vegetative growth. Ascospores are formed in a cluster of four to eight spores, which germinate and give rise to mature cells. Cells of opposite mating-types can be used to mate to form new diploids. Hybrids formed by mating of spore-derived clones have minimum gene manipulation and their use in natural

processes is therefore accepted by many as there are no conflicts with public concerns or legislation (Pérez-Través et al. 2012).

The advantage of using the mating of spore-derived clone method is that the researcher can use two parents with superior characteristics and possibly produce progeny with inherited characteristics of both parents. However, the disadvantage is that the hybrids might lose superior traits present in the parental strain due to the segregation of alleles during meiosis (Pérez-Través *et al*., 2012). Despite the 50% chance of inheriting good quality traits from both parents, it has been established that hybrids are better adapted to irregular environments, because of their innate traits.

In this study, the *HO* gene, an endonuclease gene responsible for mating-type switching of the yeast strain, will be disrupted. Thus the protoplast fusion method, although a viable option of recombining natural yeast, is redundant as this option is used when mating-type switching can occur. By disrupting the *HO* gene, the transformants remain in a haploid phase, which makes it easier to manipulate and physically mate the cells.

2.7.3 Genetic engineering

Engineering the genetic content of an organism means to alter or manipulate its genome. Over the years, genetic engineering has been successfully applied to well-known laboratory strains of *S. cerevisiae* for different reasons (Le Borgne, 2012). These reasons include extension of substrate and product range, enhancement of productivity and yield, exclusion of by-products and improvement of process performance and cellular properties. Using genetic engineering techniques scientists can add or remove specific features or characteristics from strains which allow them to create strains with new or improved characteristics.

Genetic engineering has mostly been performed on laboratory yeast strains (Le Borgne, 2012). It is therefore imperative that this technique is applied to natural *S. cerevisiae* strains especially for the use in natural applications, because these yeast are more suitable for such application due to their robust characteristics (Hahn-Hägerdal *et al*., 2001; Le Borgne, 2012).

There are various techniques by which *S. cerevisiae* strains could be genetically engineered. The most commonly used techniques for genetic engineering consist of recombinant DNA technologies, microinjection, bioballistics, electro and chemical poration (Meredith, 1982; Sridhar, 2006). Recombinant DNA technologies involve the use of plasmids and vectors to

carry the foreign gene into the host cell. The gene of interest or a part of it is replaced and the newly recombinant gene replicates as the host cell's genes are replicated.

In microinjection, a fine glass needle is used to inject the foreign DNA into the host cell. The DNA automatically enters the nucleus where it incorporates with the host cell's genetic material and replicates. With bioballistics, small silver particles are used to insert the genetic material into the host cell. The particles are coated with the foreign DNA and projected into the host cell where it incorporates with the genetic material of the host cell. This method is referred to as the shotgun method. In the electro- and chemical method, pores are created in the membrane of the cell so the genetic material can be transferred (Meredith, 1982; Sridhar, 2006).

2.7.4 Factors affecting natural strain development

The multiple ploidy, prototrophic, homothallic and often heterozygous nature of natural *S. cerevisiae* strains make them difficult to manipulate through traditional improvement strategies (Le Borgne, 2012; Volschenk *et al*., 2004). These strategies employ recombinant DNA technologies that can easily be applied to laboratory strains, which typically exist as stable haploids, display a good mating ability, easily take up foreign DNA and contain convenient auxotrophic selectable markers (Le Borgne, 2012). Therefore classical mating is often used to improve natural strains (Herskowitz, 1988).

2.7.4.1 Factors affecting classical mating

Strains of *S. cerevisiae* display two life cycle types: in the homothallic cycle, the α or **a** mating-type is converted to the opposite mating type to allow the cells to mate with each other and produce diploids. During the heterothallic cycle, the cell maintains a stable mating-type and remains in the haploid phase (Tamai *et al*., 2000). Homothallism was first described by Winge in 1935 after he observed diploid cells in a culture grown from a single haploid spore. These cells were able to sporulate, but unable to mate (Bakalinsky & Snow, 1990). Homothallism (*HO*) is conferred by a single gene on chromosome III, the *HO* gene, at the mating-type-determining (*MAT*) locus of which the dominant allele confers homothallism and the recessive allele confers heterothallism (Bakalinsky & Snow, 1990).

The *HO* gene encodes for an endonuclease that specifically cleaves the *MAT* locus and promotes interconversion of the mating-type information from the silent loci *HML*α or *HMR***a**

which are located on the telomere of chromosome III, thereby initiating mating-type switching (Bakalinsky & Snow, 1990; Kodama *et al*., 2003; Tamai *et al*., 2000; van Zyl *et al*., 1993). The α- or **a**-information encoded by *HML*α and *HMR***a** respectively, becomes mobile and translocates to the position in the *MAT* locus where the double-stranded nick has been made. Once this event takes place, the new mating-type information is expressed (Breeden & Nasmyth, 1985).

Most natural *S. cerevisiae* strains are homothallic; they can initiate spontaneous mating among them and produce non-mating diploid cells (Bakalinsky & Snow, 1990; van Zyl *et al*., 1993). The expression of the *HO* endonuclease gene can prevent the self-mating of two genetically different strains, thereby hampering mating between different strains. To prevent spontaneous self-mating of natural strains, the *HO* gene have to be inactivated. Various means of disrupting the *HO* gene has been used to create heterothallic yeast strains. For example, Van Zyl *et al*. (1993) converted homothallic *S. cerevisiae* strains to heterothallism by disrupting the *HO* gene with a disruption cassette. Tamai *et al.* (2000) and Kodama *et al.* (2003) also followed this method, whereas Schiestl (1989) treated the cells with DNA damaging agents.

2.7.4.2 The HO gene: regulation and function

The *HO* endonuclease is a homing (process of determining the location) site-specific enzyme that cleaves the mating-type locus, *MAT* and replaces it with one of the silent loci *HML*α or *HMR***a** (Figure 15). This endonuclease belongs to the LAGLIDADG family (Figure 16) as part of the Group I introns (Bakhrat *et al*., 2004; Chevalier & Stoddard, 2001; Lambowitz *et al*., 1999). It contains 586 amino acids of which the carboxyl-terminal contains five zinc fingers that have been shown to be important for the *HO* activity (Bakhrat *et al*., 2004; Katz Ezov *et al*., 2010).

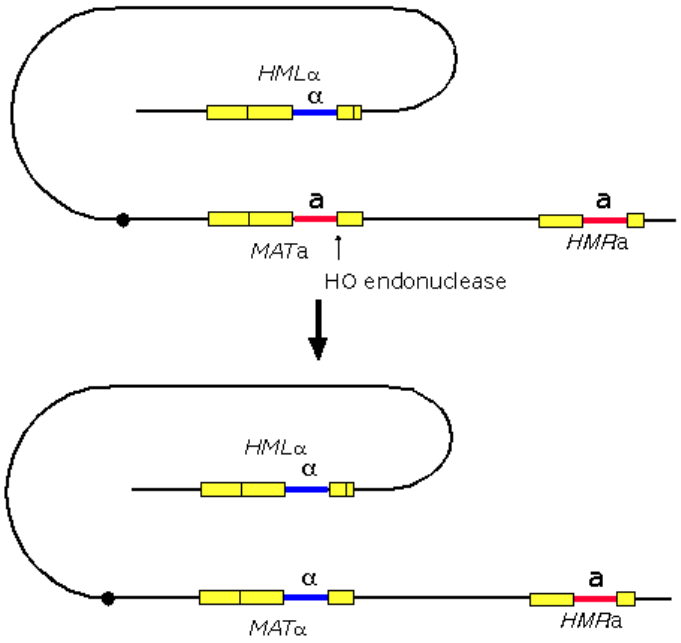

Figure 15: Gene switching induced by expression of a functional *HO* endonuclease. The *MAT* alleles can be switched with the silent loci *HML*α or *HMR***a** (www.bio.brandeis.edu).

A comparative study by Bakhrat *et al*. (2004) on homology between the homing endonucleases found that the primary sequence of the *HO* endonuclease had approximately 50% similarity to the primary sequence of the PI-*Sce*I endonuclease. The protein structure of the *HO* endonuclease was predicted based on that of PI-*Sce*I using the programme MODELLER (Figure 16).

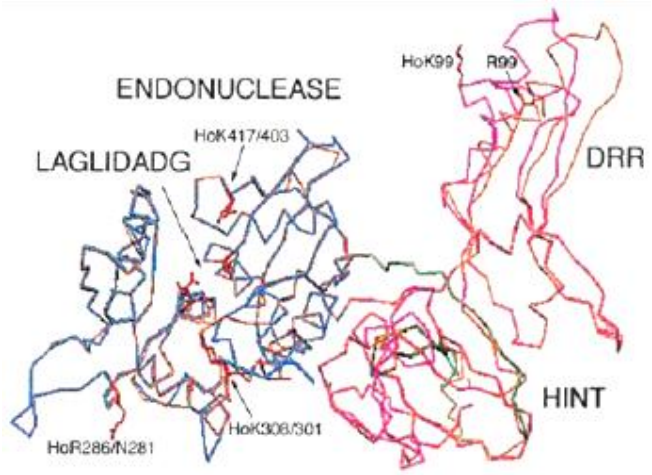

Figure 16: The *HO* endonuclease homology structure showing the LAGLIDADG domain. The Hedgehog and intein (HINT) and DNA recognition region (DRR) contains the protein splicing domain (Bakhrat *et al*., 2004).

The LAGLIDADG family has more than 200 members and is also referred to as dodecapeptides, dodecamers and decapeptides (Chevalier & Stoddard, 2001; Gimble, 2000). These endonucleases have adapted intron mobility and RNA splicing functions and were found to be encoded by free-standing genes (Bakhrat *et al*., 2004; Chevalier & Stoddard, 2001; Lambowitz *et al*., 1999). They are defined by having one or two copies of the conserved LAGLIDADG motifs. The enzymes with two copies of this motif are separated by 80 to 150 amino acids (Chevalier & Stoddard, 2001; Lambowitz *et al*., 1999). These endonucleases are the most phylogenetically diverse of the endonuclease families and are distributed in various hosts including plant genomes, algal chloroplasts, fungal and protozoan mitochondria, bacteria and archaea (Chevalier & Stoddard, 2001). Their extensive distribution can be attributed to their notable ability to invade unrelated types of intervening sequences (Chevalier & Stoddard, 2001). It has been proposed that all LAGLIDADG endonucleases recognize 14 to 30 base pairs (bp) DNA sites and cleave the DNA to generate a 4-bp 3' cohesive end (Bolduc *et al*., 2003; Gimble, 2000). These endonucleases require the presence of divalent cations as co-factors to complete their activity, which is the case with most nucleases (Chevalier & Stoddard, 2001; Gimble, 2000).

Transcriptional regulation of the *HO* gene of *S. cerevisiae* is governed by a large and complex promoter, which is comparable to that of higher classified eukaryotes. It is activated through a set of transcriptional factors and co-activator complexes that bind to the promoter and form the composite promoter (Mathias *et al*., 2004). The expression of the *HO* gene occurs during the interphase of the cell cycle. The *HO* gene product leads to the switching in mating-type which is confined to the mother cell. The mother cell is therefore the only cell that can switch mating-type during vegetative growth (Ezov *et al*., 2010; Haber, 1998).

Mating-type switching relies on four phenomena: (a) half of the cell population switch mating-type at a certain point in time, usually in late G1 phase, (b) the two silent loci's (*HML*α and *HMR***a**) act as donors during the switching event, (c) a site-specific double-stranded nick that induces recombination at *MAT*, which results in the substitution of the a or α sequences, and (d) a mechanism that regulates the use of the two donors (Haber, 1998). After switching has occurred, mating of opposite mating-type cells takes place within the same colony. The *MAT***a** / *MAT*α diploids that are formed are sterile. Both *MAT* alleles are co-dominant and the *HO* endonuclease gene is shut down (Haber, 1998; Klar, 2010). By

disrupting or mutating the *HO* gene the yeast changes from being homothallic to heterothallic. Heterothallic yeasts display a stable haploid phase with a stable mating-type.

2.8 Conclusion

Fuel is an essential part of modern living as it is used in the generation of electricity, the transport sector and the farming sector to operate machinery to produce food crops (Balat, 2011; Dias *et al.*, 2009; Escobar *et al.*, 2009; Nigam & Singh, 2011). Fossil fuels are no longer a stable source of fuel supply (Atilgan & Azapagic, 2014). The WEC predicted that this source will dry up in 2030, but the current extensive use of fossil fuels will deplete this source before the predicted cut-off date (Atilgan & Azapagic, 2014; Shafiee & Topal, 2009). Apart from this source not being a stable supply, the increase in fuel prices and the environmental hazard of fossil fuels has led to a worldwide consensus decision to search for alternative fuels (Azadi *et al*., 2012; Escobar *et al*., 2009).

Biofuels is a strong candidate to replace fossil fuels due to its environmentally friendly status, it can be produced from cheap substrates and it does not disturb the food supply chain (Macedo *et al*., 2008; Nigam & Singh, 2011; Subhadra & Edwards, 2010). Biofuels are divided in different categories such as bio-ethanol, bio-diesel, bio-methanol and bio-hydrogen (Demirbas, 2007; Nigam & Singh, 2011). Bio-ethanol is a reliable substitute to petroleum and can be used as an ethanol-petroleum blend. Economical and technical barriers however, hinder certain processes within the production of bio-ethanol. One economical barrier is the expensive nature of the commercial enzymes that are needed to break down the sugar polymers within the substrate (lignocellulose) to produce simple sugar units. Technical barriers include the crystallinity of the substrate that requires an additional pre-treatment step to expose the sugar polymers for following steps in the production process. Another obstacle is the use of a suitable microorganism to convert the sugar units to ethanol.

S. cerevisiae, however, has been identified as a suitable microorganism to produce bio-ethanol on an industrial scale. Natural *S. cerevisiae* strains that display all the necessary characteristics such as fermentation vigour, ethanol tolerance, inhibitor tolerance, osmotolerance, thermotolerance and a strain that has the ability to consume a wide range of substrates has not yet been identified. This study, therefore, focuses on the use of the mating of spore-derived clones to generate hybrid progeny. This method has the advantage of physically mating the resultant clones of the parental strains that display different

characteristics. Applying the method has a benefit, as the progeny have a better chance of inheriting both parental characteristics.

The *HO* gene of the parental *S. cerevisiae* strains had to be disrupted in order to obtain them in a stable haploid phase so as to prevent the self-mating. Natural *S. cerevisiae* strains are usually diploid or polyploid and because of a functional *HO* gene, have the ability to switch mating-types and self-mate. This allows for no or little variation in the genetic profile of the resulting hybrids as the hybrids display the characteristics of the original parent. However, during the mating of spore-derived clones, clones from two parents displaying complementing characteristics are combined to create superior diploid hybrids.

2.9 Relevance of the study

A robust *S. cerevisiae* strain with all the desired characteristics (ethanol tolerance, inhibitor tolerance, osmotolerance, good fermentation vigour and temperature tolerance) has not yet been identified. This study focuses on obtaining hybrid *S. cerevisiae* CBP host strains with desired traits through the mating of spore-derived clones. Generating such a strain could be valuable especially for the bio-ethanol industry as the need for alternative fuels is growing. The market for bio-ethanol is also on the verge of expansion and provides opportunities for the use of superior *S. cerevisiae* strains that could be used in the production of bio-ethanol on an industrial scale. The study was therefore undertaken with the aim of generating hybrid *S. cerevisiae* strains with desired traits.

Chapter 3: Materials and Methods

3.1 Yeast strains

Four strains (HR14, YI64, YI2 and MF15) obtained from the culture collection of the Agriculture Research Council (ARC) Infruitec-Nietvoorbij (Stellenbosch, South Africa) were selected for this study based on the following characteristics (Mrs. Trudy Jansen): YI64 displayed good fermentation capability, grew at 40°C and had a high osmotolerance. HR14 was inhibitor tolerant, YI2 displayed good fermentation ability and could grow in the presence of 20% ethanol. MF15 was osmo- and inhibitor tolerant.

3.2 Ploidy determination

The four yeast strains were sporulated on sporulation agar (1% potassium acetate, 1.5% bacteriological agar) to determine the ploidy (adapted from Fast, 1973). An incubation period of two weeks at room temperature was allowed for ascospore formation.

3.3 Antibiotic resistance

The strains were plated onto Yeast Peptone Dextrose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose, 1.5% bacteriological agar) supplemented with either 50 - 200 µg/ml Geneticin® or 50 - 1000 µg/ml Zeocin®. The plates were incubated at 30°C for 3 - 5 days to determine the minimum growth inhibition concentration (MIC) for each strain (Andrews, 2001; Islam *et al*., 2008; Yörük & Albayrak, 2015).

3.4 Construction of disruption cassette

The *kanMX* DNA disruption cassette (Figure 17 A) was obtained using PCR with plasmid pBKD1 (Mascoma Corp.) as template and *kanMX* forward and reverse primers that contained 140 bp overhangs of the 5' and 3' ends of the *HO* gene (*Saccharomyces cerevisiae* Genome Database) (Kodama *et al*., 2003; Russell *et al*., 1986). Similarly, the *Sh ble* DNA disruption cassette (Figure 17 B), containing 140 bp *HO* flanking regions at the 5' and 3' ends, was obtained using plasmid pBZD2244 (Mascoma Corp.) as template and the *Sh ble* forward and reverse primers. The relevant primer sequences are listed in Table 1.

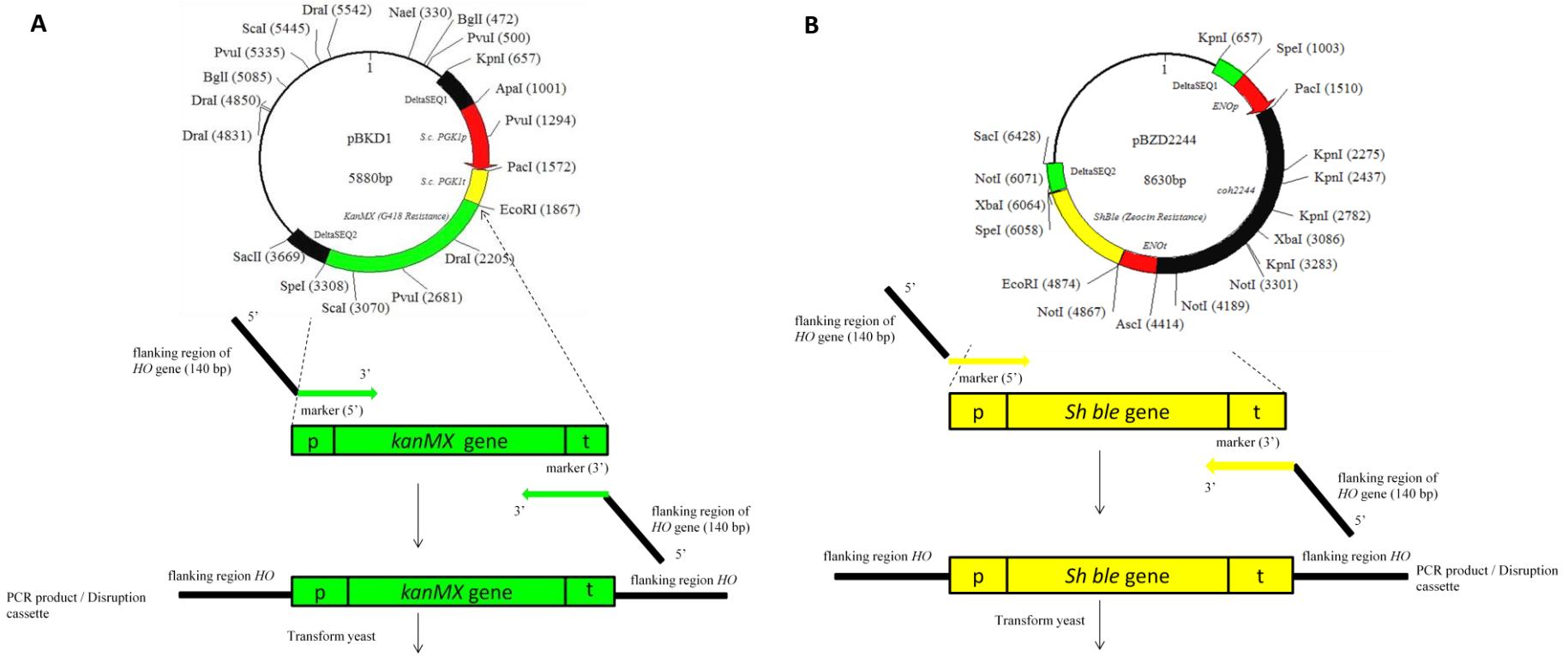

Figure 17: Construction of the *kanMX* (A) and *Sh ble* (B) disruption cassettes.

Table 1: List of primers used in this study

Primer names	**Primer sequences**
kanMX forward	5'**AAAACGCTTCATAGAAGAAATGGAGCGCTCTAAAGGAGAATATTTCAACTTTGACATTGAAGTTAGAGATTTGGATTATCT TGATGCTCAATTGAGAATTTCTAGCTGCATAAGATTTGGTCCAGTACTCGCAGGAAATG**GAATTCGTTTAGCTTGCCTCG'3
kanMX reverse	5'**TATACGGGTTCCCTTTTTATAATTGGCGGAACTTCTCTTGTTTTGTGACCACTTCGACAATATGACAAAACATTCTGTGAAG TTGTTCCCCCAGCAACATTACAGTCGTATGTAAATTGACATTGGACTTTTCTTCCTTC**TTTTCGACACTGGATGGCG'3
Sh ble forward	5'**GGCTTGGATGCTTGGTCTGTGGTTAGGTGACAGTACAACAAAAGAGCCAGAAATCTCAGTAGATAGCTTGGATCCTAAGCT AATGGAGAGTTTAAGAGAAAATGCGAAAATCTGGGGTCTCTACCTTACGGTTTGTGACG**GAATTCCCCACACACCATAGC'3
Sh ble reverse	5'**GCAACACAGTGTTTTAGATTCTTTTTTTGTGATATTTTAAGCTGTTCTCCCACACAGCAGCCTCGACATGATTTCACTTCTA TTTTGTTGCCAAGCAAGAAATTTTTATGGCCTTCTATCGTAAGCCCATATACAGTACT**ACTAGTAATTCAGCTTGCAAATTAAA GC'3
HO forward primer	5'GATCAAGCTTATGCTTTCTGA'3
HO reverse primer	5'GATCGAATTCTTAGCAGATGC'3
*MAT***a** forward primer	5'ACTCCACTTCAAGTAAGAGTTTG'3
MAT locus overlap primer	5'AGTCACATCAAGATCGTTTATGG'3
*MAT*α reverse primer	5'GCACGGAATATGGGACTACTTCG'3

Bold sequences indicate sequence homologous to the *HO* gene.

The Perkin Elmer Gene Amp® PCR System 2400 (The Perkin-Elmer Corporation, Norwalk, CT, USA) and TaKaRa Ex Taq™ polymerase (Takara Bio Inc, Japan) were used for all amplification reactions. PCR amplification was performed in 50 µl reaction mixtures (50 ng plasmid DNA template, 1x Ex Taq buffer, 0.2 mM dNTPs, 1 µM of each primer, 2.5 U Ex Taq polymerase). The PCR conditions for both the *kanMX* and *Sh ble* cassettes: denaturation at 94°C for 5 minutes, 30 amplification cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min) and a final extension for 7 min at 72°C. The amplification products were visualised on a 0.8% agarose gel and the required DNA bands were excised and eluted from the agarose gel with the Zymoclean™ Gel Recovery Kit (Inqaba Biotech).

3.5 Yeast transformation

The *S. cerevisiae* YI64 and HR14 strains were transformed with both the *kanMX* and *Sh ble* disruption cassettes, whereas the *S. cerevisiae* YI2 and MF15 strains were transformed with the *Sh ble* and *kanMX* disruption cassettes, respectively (Table 2). All transformations were done using electroporation (Cho *et al*., 1999). Transformants were selected on YPD agar plates that contained the appropriate antibiotic. Integration of the disruption cassettes was verified by PCR amplification with genomic DNA (Hoffman & Winston, 1987) as template. The PCR reactions and conditions were (500 ng gDNA template, 1x Ex Taq buffer, 0.2 mM dNTPs, 1 µM of each primer, 2.5 U Ex Taq polymerase): denaturation 94°C for 5 minutes; amplification 30 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min) and extension 72°C for 7 min. The products were visualised on a 0.8% agarose gel.

Table 2: Disruption of the *HO* gene in the *S. cerevisiae* strains

Strain	**Marker-gene**	**Antibiotic concentration**	**Description**
HR14	*kanMX* *Sh ble*	100 µg/ml 50 µg/ml	Double disruption**
YI64	*kanMX* *Sh ble*	100 µg/ml 500 µg/ml	Double disruption**
YI2	*Sh ble*	600 µg/ml	Single disruption*
MF15	*kanMX*	100 µg/ml	Single disruption*

*Single *HO* allele disrupted **Both *HO* alleles disrupted

3.6 Sporulation and spore dissection

The disrupted transformants were allowed to sporulate on sporulation agar for two weeks at room temperature. Asci were suspended in 2 ml sterile double distilled water (ddH_2O) and washed twice (centrifuged at 5000 rpm for 5 minutes and resuspended in 500 µl ddH_2O). Asci were resuspended in 150 µl lysis buffer (0.5 mg/ml Zymolase in 1 M sorbitol) and incubated overnight in a 30°C waterbath. The spores were washed with 2 ml ddH_2O and the pellets resuspended in 500 µl ddH_2O. The Singer MSM System 200 micromanipulator microscope (Singer Instruments, Somerset, England) was used to dissect the spores on YPD plates containing the appropriate antibiotic (Geneticin® / Zeocin®) and incubated at 30°C for a period of 2 - 3 days to germinate. Spore-derived clones were streaked out three times and allowed to grow for 2 - 3 days.

3.7 Mating

S. cerevisiae haploid strains with opposite mating-types were used in the mating experiments (Pérez-Través *et al*., 2012; Rainieri & Pretorius, 2000). The clones were mated on YPD plates and allowed to grow overnight. The diploid progeny was confirmed with PCR. The PCR primers (Table 1) were designed based on the open reading frames of the *MAT*a, *MAT*α and *MAT* locus genes (Huxley *et al*., 1990). PCR amplification was performed in 25 µl reaction mixtures [500 ng gDNA as template, 1x Ex Taq buffer, 0.2 mM dNTPs, 1 µM of each primer (*MAT*a, *MAT*α, *MAT* locus), 2.5 U Ex Taq polymerase] and the following PCR conditions: denaturation at 94°C for 5 minutes, followed by 30 amplification cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and a final extension at 72°C for 7 min. The amplification products were visualized on a 0.8% agarose gel.

The genomic stability of the best performing hybrid strains was evaluated; strains were inoculated into 5 ml YPD broth (without antibiotics) and incubated on the test tube wheel at 30°C until growth reached stationary phase (determined spectrophotometrically at 600 nm). Five microliters of each culture was transferred daily (in triplicate) to fresh YPD medium; this was repeated 10 times before the cultures were plated onto YPD agar containing either Geneticin® or Zeocin® and incubated at 30°C for 3 - 5 days.

3.8 Southern blot analysis

Genomic DNA of the transformed strains was isolated (Hoffman and Winston, 1987) and digested overnight with *Pvu*II at 37°C. The products were separated on a 0.8% agarose gel,

transferred to a positively charged nylon membrane (Bio-Rad Laboratories Ltd.) and exposed to a labelled *HO* probe. For the latter, the *HO* gene of the YI64 parental strain was amplified with PCR using the *HO* forward and reverse primers (Table 1) (*Saccharomyces* Genomic Database). The *HO* gene PCR amplifications was performed in 25 µl reaction mixtures [500 ng gDNA as template, 1x Ex Taq buffer, 0.2 mM dNTPs, 1 µM of each primer, 2.5 U Ex Taq polymerase] using denaturation at 94°C for 5 minutes, followed by 30 amplification cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and a final extension at 72°C for 7 min. The amplification products were visualised on a 0.8% agarose gel followed by labelling and detection using the digoxigenin (DIG) DNA Labelling and Detection kit (Roche Ltd).

3.9 Anaerobic fermentations

Pre-cultures were prepared by growing yeast strains overnight in 5 ml synthetic nutrient (MNS) medium (0.1 g $CaCl_2$, 0.1 g $NaCl_2$. 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 3 g tartaric acid, 0.3 g $(NH_4)_2SO_4$, 0.3 g $(NH_4)_2HPO_4$, 200 g glucose, 0.2 g casein hydrolysate, 2 g malic acid, 0.2 mg $NaMoO_4.2H_2O$, 0.4 mg $ZnSO_4.7H_2O$, 0.5 mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 0.1 mg KI, 0.4 mg $FeCl_3.6H_2O$, $MnSO_4.H_2O$, 0.4 mg pyridoxine chlorohydrate, 0.4 mg thiamine chlorohydrate, 2 mg inositol, 0.02 mg biotin, 0.4 mg calcium pantothenate, 0.4 mg nicotinamide, 0.2 mg p-amino benzoic acid, per litre) (Delfini, 1995). Overnight cultures were inoculated in triplicate into glass serum bottles containing 100 ml MNS medium at an average cell concentration of 7.5 x 10^4 cells/ml. The glass bottles were sealed with rubber stoppers and crimp vial caps. Each bottle was fitted with a magnetic stirrer bar, as well as two syringe needles, one attached to a 2 ml syringe for sampling and one as a vent for CO_2. The 100 ml fermentation vessels were incubated on magnetic stirrers at 30°C and monitored for 7 - 14 days. Fermentation ability was monitored daily by measuring cell growth and glucose- and ethanol concentration.

Cell growth was quantified using a spectrophotometer (absorbance at 600 nm) or haemocytometer (number of cells) and standard curves were used to determine the relationship between absorbance and cell counts. All growth curves and cell count estimations were done in triplicate. Glucose and ethanol concentrations were quantified with high performance liquid chromatography (HPLC) using a Surveyor Plus liquid chromatograph (Thermo Scientific) consisting of a LC pump, autosampler and refractive index detector. The compounds were separated on a Rezex RHM Monosaccharide 7.8 x 300 mm column (00H0132-K0, Phenomenex) at 60°C with 5 mM H_2SO_4 as mobile phase at a

flow rate of 0.6 ml/min. All HPLC data were analysed using the Statistical Analysis System (SAS) software (SAS Institute Inc., 1999). Briefly, the data was tested for normality (Shapiro & Wilk, 1965) and showed a normal distribution, after which the analysis of variance (ANOVA) was applied. Student's *t* least significant difference (LSD) was calculated at the 5% significance level to facilitate comparison between strains.

3.10 Inhibitor and ethanol tolerance

Anaerobic fermentations were set-up in triplicate as described above with the addition of a 25% synthetic inhibitor cocktail (19 mM formic acid, 19 mM acetic acid, 7.5 mM furfural, 7.5 mM HMF, 0.25 mM coniferyl aldehyde, 0.25 mM cinnamic acid) to the culture medium. To evaluate ethanol tolerance, similar fermentations were set-up in triplicate with the addition of 10% ethanol (v/v). Fermentation ability was monitored daily by measuring cell growth (absorbance at 600 nm) glucose and ethanol concentration.

3.11 Ethanol adaptation of hybrid strains

Pre-cultures of the best performing hybrids were inoculated in 150 ml Erlenmeyer flasks containing 50 ml MNS supplemented with 5, 7.5 or 10% ethanol and incubated at 30°C on an MRC orbital shaker [United Scientific (Pty) Ltd] at 200 rpm. Adaptations to ethanol was allowed to continue for a maximum of one week. The cultures were transferred to fresh MNS supplemented with a higher ethanol concentration once the stationary phase was reached (as measured spectrophotometrically at 600 nm). Cultures were transferred three times to the same ethanol concentration to obtain stable populations, before moving to a higher concentration.

Hybrid strains were adapted to a final concentration of 10% ethanol. Fermentation experiments were set-up as above with the addition of 10% ethanol. The fermentation ability of selected strains was also assessed in the presence of triticale straw hydrolysate (0.424 g/l glucose, 0.045 g/l cellobiose, 4.405 g/l xylose, 1.121 g/l arabinose, 1.66 g/l acetic acid, 0.305 g/l formic acid, 0.481 g/l furfural and 0.523 g/l hydroxymethyl furfural). The triticale straw hydrolysate was supplemented to a final concentration of 2% glucose, due to the low glucose concentration in the hydrolysate. Triplicate fermentations were set-up as previously described and monitored daily by measuring cell growth (absorbance at 600 nm), glucose and ethanol concentration as described above.

3.12 Thermo- and osmotolerance

Parental, unadapted and ethanol-adapted hybrid strains were screened for their ability to grow at temperatures higher than 30°C. The strains were plated onto YPD agar and incubated at 40, 42, 43 and 44°C for 2 - 3 days or until stationary phase was reached. A similar approach was used to determine the osmotolerance of the strains on YPD agar plates containing 50, 55, 60 or 65% glucose after incubation for 2 - 3 days at 30°C.

Chapter 4: Results and Discussion

4.1 Characterisation of parental strains

Natural strains normally display low sporulation ability, which makes them difficult to manipulate. All four parental strains (HR14, YI64, YI2 and MF15) produced ascospores after two weeks on sporulation agar. The presence of asci was confirmed microscopically (Figure 18), with four distinctive spores per asci in all four parental strains that confirmed their diploid status.

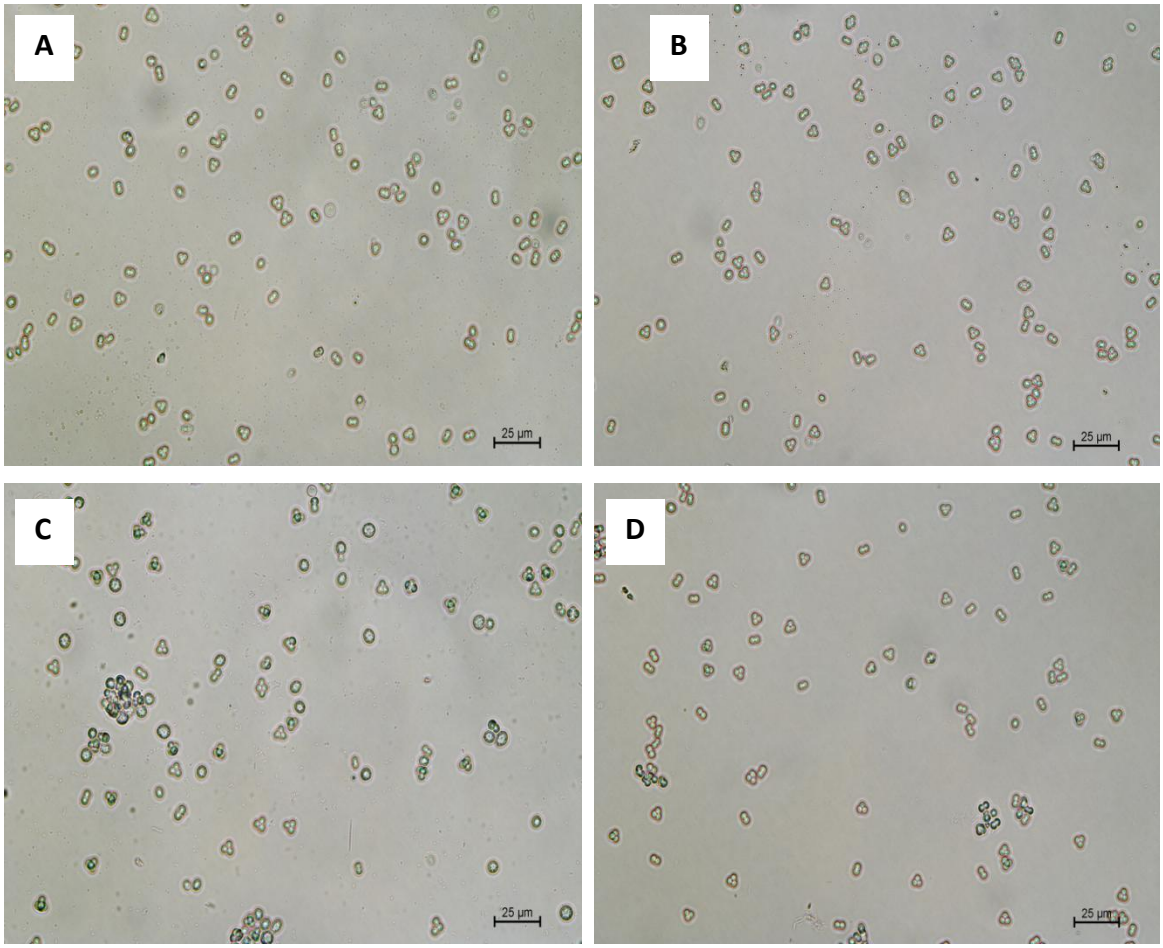

Figure 18: Asci formed by the natural *S. cerevisiae* strains (A) HR14, (B) YI64, (C) YI2 and (D) MF15.

When the natural *S. cerevisiae* strains were cultivated in YPD supplemented with Geneticin®, a minimum growth inhibition concentration (MIC) of 100 µg/ml Geneticin® was recorded for all four strains. In contrast, the MIC for Zeocin® varied between 200 - 700 µg/ml (Table 3).

Table 3: Minimum growth inhibition concentration (MIC) of *S. cerevisiae* strains

Strains	Antibiotic	Concentration range (µg/ml)	MIC (µg/ml)
HR14	Geneticin®	50 – 200	100
YI64	Geneticin®	50 – 200	100
MF15	Geneticin®	50 – 200	100
YI2	Geneticin®	50 – 200	100
HR14	Zeocin®	50 – 1000	200
YI64	Zeocin®	50 – 1000	600
MF15	Zeocin®	50 – 1000	700
YI2	Zeocin®	50 – 1000	600

4.2 *HO* disruption

Increased resistance to both Geneticin® and Zeocin® can be conferred by the transformation of *S. cerevisiae* with a plasmid or expression cassette that contains the dominant selectable markers *kanMX* and *Sh ble* respectively. Two gene-specific disruption cassettes were designed that contained either the *kanMX* or *Sh ble* selectable marker, flanked by 140 base pairs of the *HO* gene. As shown in Figure 19, the *kanMX* disruption cassette resulted in a PCR product of 1 722 bp (1 582 bp *kanMX* gene + 140 bp *HO* region) and the *Sh ble* disruption cassette a product of 1 470 bp (1 330 bp *Sh ble* gene + 140 bp *HO* region).

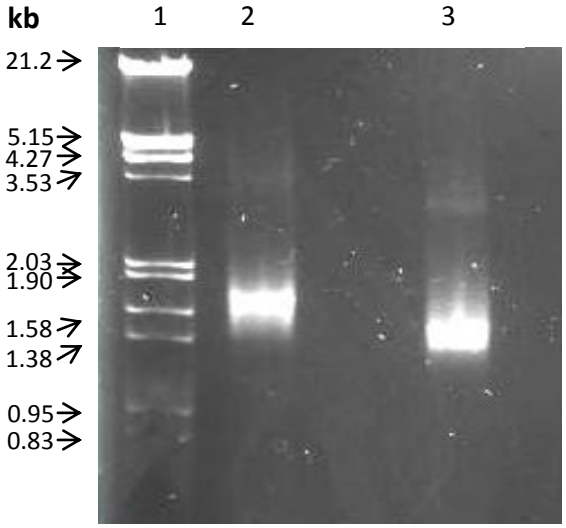

Figure 19: Agarose gel electrophoresis of the amplified disruption cassettes. Lane 1: DNA marker is λ DNA cleaved with EcoRI and HindIII (λ III with sizes depicted on the left hand side). Lane 2: *G418* disruption cassette. Lane 3: *Sh ble* disruption cassette.

The *S. cerevisiae* YI64 and HR14 strains were transformed with both *kanMX* and *Sh ble* disruption cassettes, whereas the *S. cerevisiae* YI2 and MF15 strains were transformed with the *Sh ble* and *kanMX* disruption cassettes, respectively. All the transformed strains produced four-spored asci after two weeks on sporulation agar. Single spores were isolated from each strain and a total of 64 clones were obtained: HR14 produced 27 clones, YI64 five clones, MF15 twenty clones and YI2 twelve clones.

The mating-type of the spore-derived clones was determined by amplification of the *MAT* loci, with expected PCR products of 544 and 404 bp for the *MAT***a** and *MAT*α mating types, respectively. Forty-six of the 64 spore-derived clones were still diploid and therefore disregarded. Twelve clones from HR14, YI64 and MF15 were *MAT***a** and 6 clones from HR14, MF15 and YI2 were *MAT*α.

Strains with the opposite mating-type were used in the mating experiments, but not all mating experiments produced viable diploids. Mating of spore-derived clones from strains YI64 (*MAT***a**) and HR14 (*MAT*α) produced 3 YH clones, all of which (YH1, YH2 and YH3) were selected for further analysis (Figure 20). Mating of spores-derived clones from strains MF15 (*MAT***a**) and YI2 (*MAT*α) produced 16 MY clones, of which 3 representatives, MY3, MY5 and MY7 were selected for further analysis.

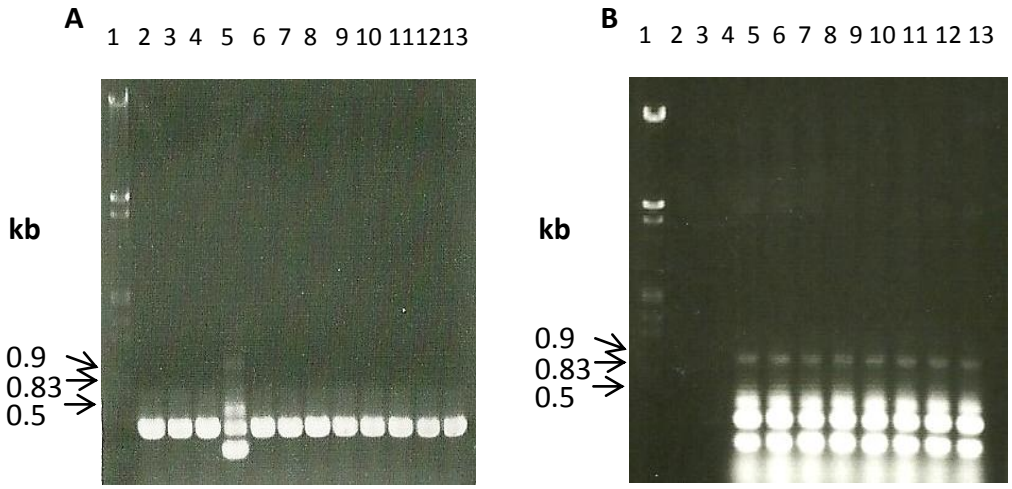

Figure 20: Agarose gel electrophoresis of PCR products obtained for the *MAT***a**/α PCR of the hybrids. (A) Hybrids obtained from mating experiments between the spores obtained from the *S. cerevisiae* YI64 and HR14 strains; Lane 5 represents: YH1 hybrid strain. (B) Hybrids obtained from mating experiments between the spores from the *S. cerevisiae* MF15 and YI2 strains. Lane 7: hybrid strain MY3; Lane 9: hybrid strain MY5; Lane 11 hybrid strain MY7.

The *HO*-disruption was confirmed by amplification of the disruption cassettes. The *HO* gene is 1 761 bp in size, therefore amplicons of 2 402 bp and 2 625 bp were expected for the *HO-Sh ble* and *HO-G418* disruption cassette, respectively. Figure 21 shows the intact *HO* gene of the parental strains (± 1 700 bp), as well as *HO* disruptions for the hybrid strains YH3 and MY5, indicated by a larger amplification product. This confirmed that electroporation provides an effective method to achieve inactivation of yeast genes through targeted deletion-disruption strategies.

Southern blot analysis of the genomic DNA of the four *S. cerevisiae* parental strains (HR14, YI64, MF15 and YI2) and the YH3 and MY5 hybrid strains using the *S. cerevisiae HO* gene as probe confirmed the successful disruption of the *HO* gene (Figure 22). An intact *HO* gene of 1 761 bp was observed in the parental strains, whereas the size of the *HO* fragment in the YH3 and MY5 hybrid strains was 2 625 bp (*G418*) and 2 402 bp (*Sh ble*). The results confirm the replacement of the wild-type *HO* alleles with the respective disruption cassettes at the *MAT* locus. It also confirms that homologous recombination transpired during the transformation process and that subsequent meiotic segregation of the disrupted *HO* alleles in the tetrad progeny occurred.

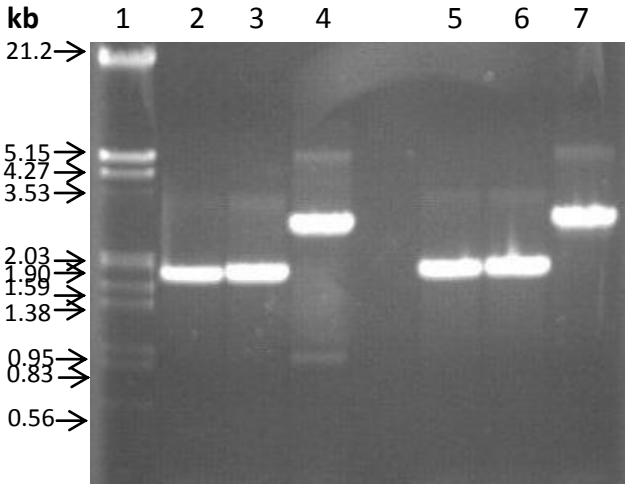

Figure 21: Agarose gel electrophoresis of the *S. cerevisiae HO* gene PCR products. Lane 1: DNA marker λIII with the sizes indicated on the left hand side. Lane 2: parental strain YI64; Lane 3: parental strain HR14; Lane 4: hybrid strain YH3; Lane 5: parental strain YI2; Lane 6: parental strain MF15; Lane 7: hybrid strain MY5.

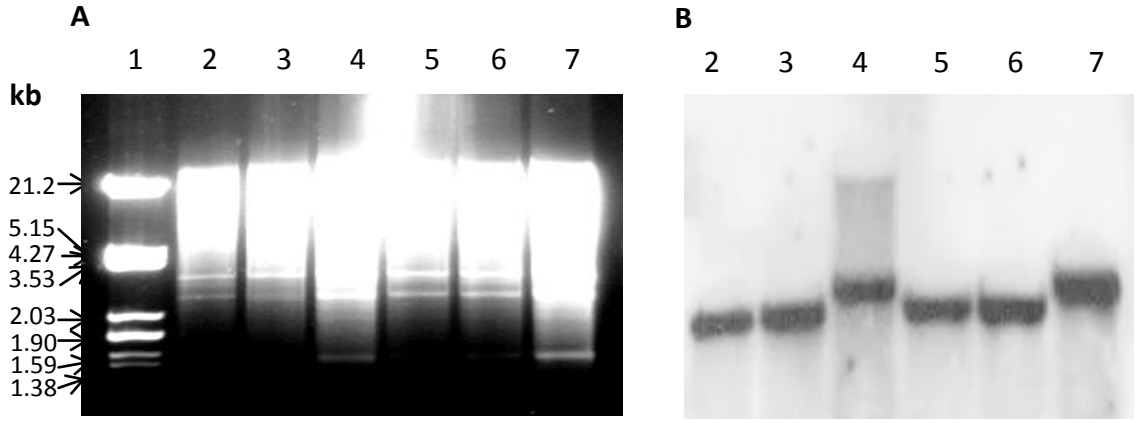

Figure 22: (A) Digested genomic DNA and (B) the Southern blot analysis of the parental strains. Lane 1: DNA Marker λIII; Lane 2: YI64; Lane 3: HR14; Lane 4: YH3; Lane 5: YI2; Lane 6: MF15; Lane 7: MY5.

4.3 Fermentation characteristics

The fermentation characteristics and ethanol production of the HR14 and YI64 parental strains were compared with the YH1, YH2 and YH3 hybrid strains in terms of fermentation vigour, osmotolerance, ethanol tolerance and inhibitor tolerance. The YI64 parental strain confirmed results (good fermentation vigour) from a previous screening done by Mrs. Trudy Jansen (unpublished data) and YI64 indicated good fermentation capabilities (Figure 23). Strain YI64 showed a faster glucose consumption rate and higher ethanol production levels than strain HR14. The fermentation vigour of the YH1 and YH2 hybrid strains was low relative to the two parental strains, whereas the YH3 hybrid strain was more comparable to the parental YI64 strain, producing almost 100 g/L ethanol by day 7.

Statistical analyses of the data indicated that the ANOVA p-values for both glucose consumption and ethanol production were less than 0.05, i.e. there is a significant difference between the data produced by the different strains. The mean values of the t-Test (Table 4) obtained for all the strains implies that the strains performed differently in terms of their glucose consumption and ethanol production. The YI64 parental and YH3 hybrid strains performed similarly in both glucose consumption and ethanol production. These two strains also produced the highest level of ethanol during this fermentation trail, evident in the mean values of the t-Test (Table 4) which means that these two strains performed significantly better in their glucose consumption and ethanol production than the other three strains.

Previous screening of the parental strains (Trudy Jansen, unpublished) indicated that the YI2 parental strain was a better fermenter than MF15 (i.e. better glucose consumption). The current study confirmed that the YI2 parental strain performed better as it consumed glucose faster than MF15, and also outperformed all three hybrid strains, MY3, MY5 and MY7 (Figure 23). The MY3, MY5 and YI2 strains consumed most of their glucose by day 6 and produced approximately 81 g/L ethanol by day 7, whereas MF15 and MY7 strains still had residual glucose left by day 7 and produced less than 80 g/L and 70 g/L ethanol, respectively.

The p-values for glucose consumption were greater than 0.05 (i.e. no significant differences in the data) and less than 0.05 for ethanol production (i.e. significant differences). The mean values of the t-Tests (Table 4) also confirmed that YI2, MY3 and MY5 had similar glucose consumption rates, but no significant difference in ethanol production could be detected between the strains. The mean values in Table 5 also show a significant difference for YI2 strain, as this strain was the best ethanol producer and consumed glucose much faster than the other strains (Figure 23).

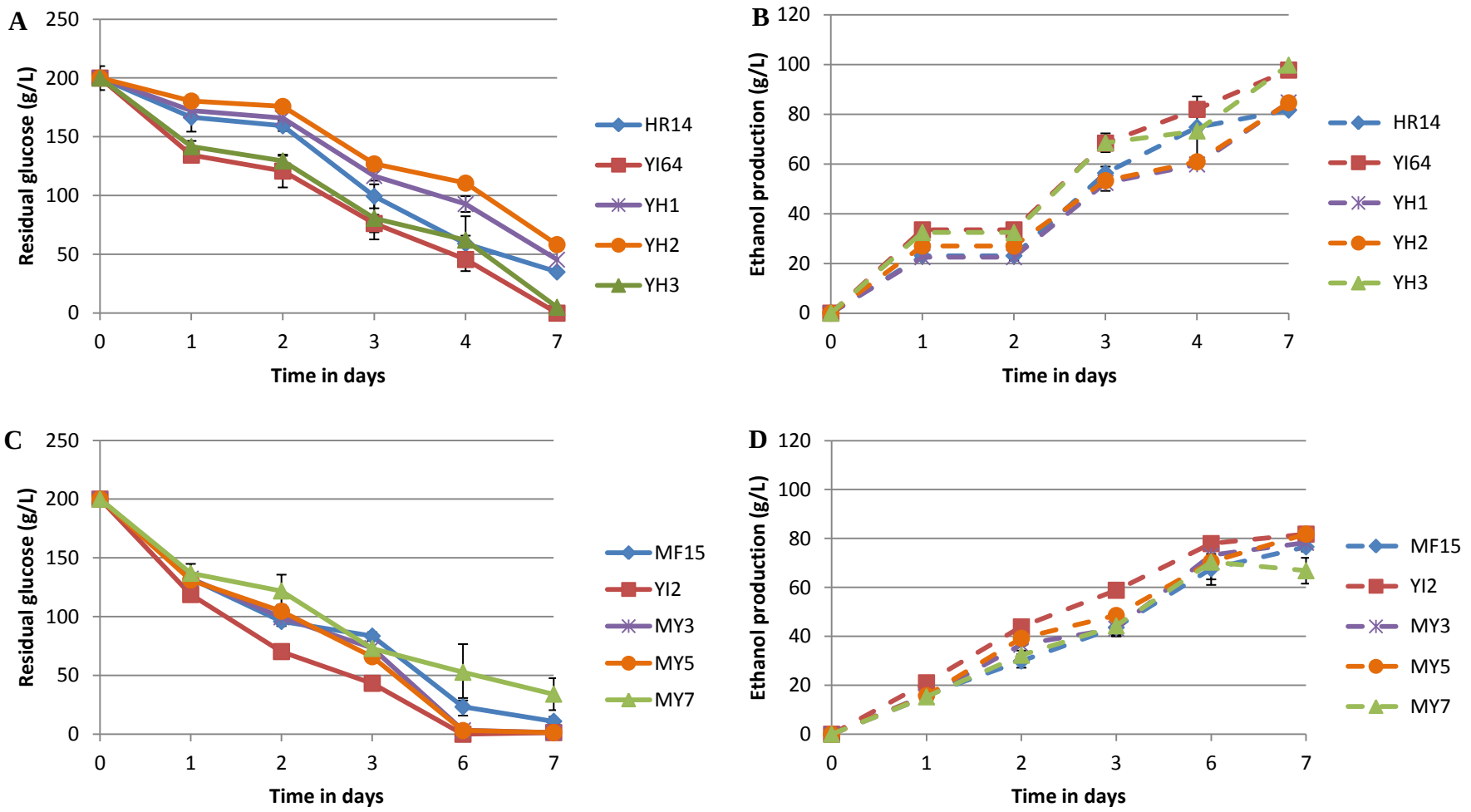

Figure 23: Residual glucose (—) and ethanol production (--) by *S. cerevisiae* strains in MNS medium. HR14 and YI64 parental and hybrid strains (A and B). MF15 and YI2 parental and hybrid strains (C and D).

Table 4: Mean residual glucose (g/L) and ethanol yields (g/L) by yeast strains over seven days

Strains	N	Glucose	Ethanol	Strains	N	Glucose	Ethanol
HR14	18	119.927c	43.152b	MF15	18	91.044ab	38.974b
YI64	18	96.221d	52.496a	YI2	18	72.319b	47.252a
YH1	18	132.193 b	40.319b	MY3	18	84.984ab	41.074b
YH2	18	142.039a	42.109b	MY5	18	84.384ab	42.642ab
YH3	18	103.166d	51.061a	MY7	18	103.090a	38.188b
LSD (p=0.05)		9.1757	4.9516	LSD (p=0.05)		20.457	5.7348

Values in the same column followed by the same letter indicate no significant difference. Least Significant Difference (LSD). N refers to the number of replicates multiplied by the total readings taken for each strain.

In this fermentation trail, the two parental strains, YI64 and YI2, and hybrid strains YH3 and MY5 performed better than the other strains; they displayed good fermentation vigour and produced significant levels of ethanol. The performance of YI64 confirmed the initial finding by Trudy Jansen (unpublished) that it is a good fermenter, producing almost 100 g/L ethanol by day 7 and utilizing all the available glucose. The YH3 strain showed a similar fermentation pattern to YI64 and it can be concluded that this strain is also a good fermenter. The YI2 and MY5 strains utilised all the available glucose by day 6 and can be considered as good fermenters as they produced approximately 81 g/L ethanol by day 7. The ANOVA and t-Test results also confirmed that these strains are significantly better than the other strains.

4.4 Inhibitor tolerance

Figure 24 shows the fermentation characteristics of the HR14 and YI64 parental and hybrid strains (YH1, YH2 and YH3) in the presence of 25% inhibitor cocktail. There was no significant utilisation of glucose in the presence of the synthetic inhibitor cocktail and none of the strains had the ability to produce ethanol. The inability to ferment glucose in the presence of 25% inhibitor cocktail could be due to the multiple negative effects of the inhibitors on the cell, which include retardation of growth, cell wall damage, DNA damage, inhibition of protein and RNA synthesis, etc. (Almeida *et al*., 2009; Hasunuma & Kondo, 2012; Keating *et al*., 2006). The p-values for both glucose consumption and ethanol production were greater than 0.05, which implies that there was no significant difference between the data for both glucose consumption and ethanol production.

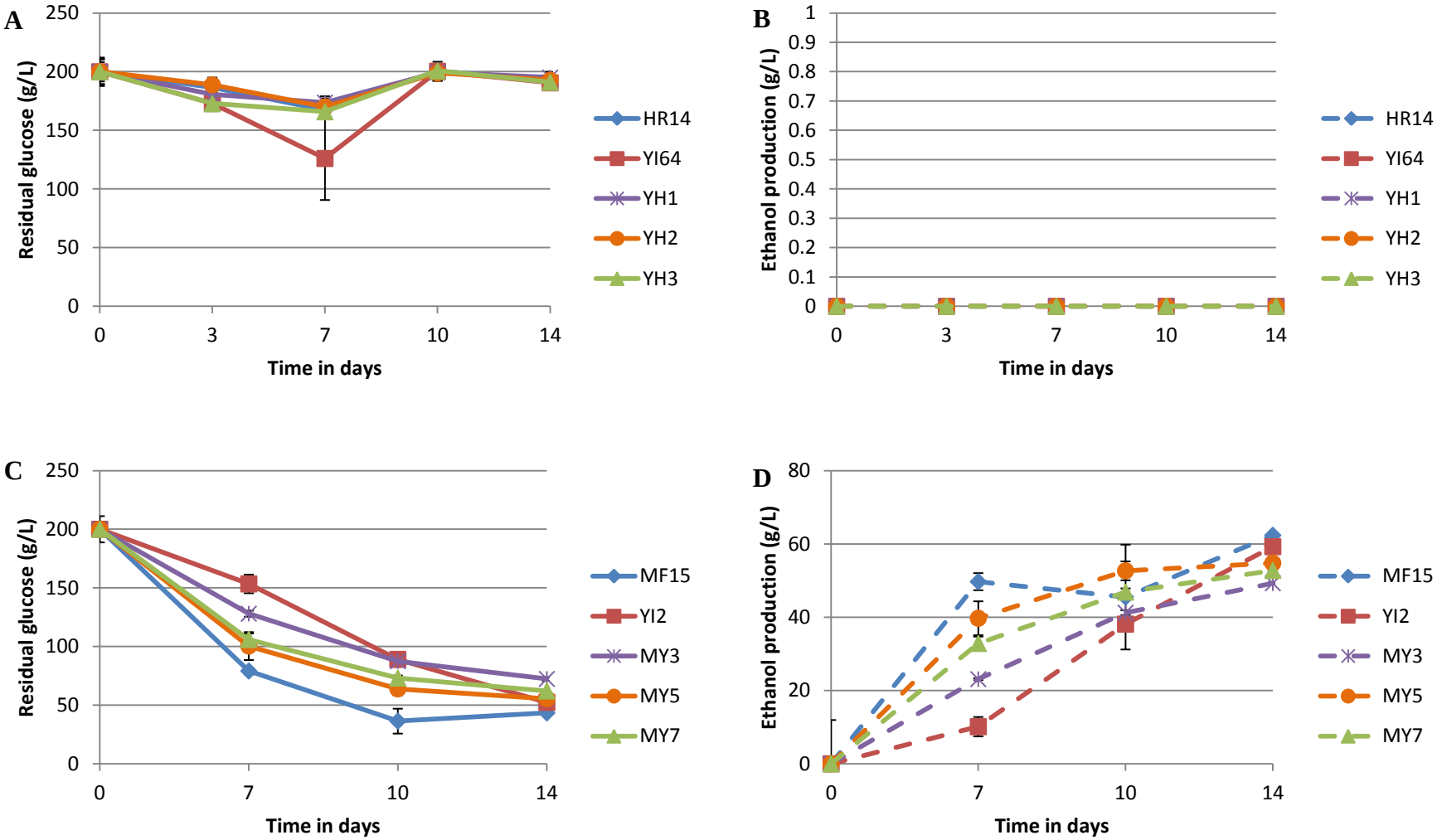

Figure 24: Residual glucose (━) and ethanol production (--) by *S. cerevisiae* strains in MNS medium containing 25% synthetic inhibitor cocktail. HR14 and YI64 parental and hybrid strains (A and B). MF15 and YI2 parental and hybrid strains (C and D).

Table 5: Mean residual glucose (g/L) and ethanol production (g/L) by yeast strains over fourteen days

Strains	N	Glucose	Ethanol	Strains	N	Glucose	Ethanol
HR14	15	191.691a	0a	MF15	12	89.758d	39.388a
YI64	15	178.993a	0a	YI2	12	123.710a	26.878b
YH1	15	189.759a	0a	MY3	12	121.978ab	28.399b
YH2	15	190.116a	0a	MY5	12	104.969c	36.785a
YH3	15	187.103a	0a	MY7	12	110.311bc	33.117ab
LSD (p=0.05)		15.449	0	LSD (p=0.05)		11.934	8.3429

Values in the same column followed by the same letter indicate no significant difference. Least Significant Difference (LSD). N refers to the number of replicates multiplied by the total readings taken for each strain.

In agreement with its initial characterisation as an inhibitor-tolerant strain (Trudy Jansen, unpublished), the MF15 parental strain consumed the glucose in the growth medium much faster than the other strains (Figure 24). MF15 consumed ± 150 g of glucose and produced ± 50 g/L ethanol by day 14. The MY5 hybrid strain also performed well in the presence of the 25% synthetic inhibitor cocktail with its performance correlating with that of MF15 (consumed ± 150 g glucose and produced 50 g/L ethanol). The p-values for both glucose consumption and ethanol production indicate that there was a significant difference in the data produced by the strains. The mean values of the t-Test (Table 5) also reveals differences between the data produced for both residual glucose and ethanol production. However, the t-Test values indicated that MF15 was significantly better than the other strains with respect to glucose consumption, but similar to MY5 with respect to ethanol production. These two strains can therefore be grouped together based on their ethanol production and can be regarded as more inhibitor tolerant than the other strains.

The poor performance of the YI2 parental strain and the MY3 and MY7 hybrid strains correlates with what has been stated in literature, viz. that inhibitors hinder growth and the cells go into an extended lag phase, reduces ethanol yield and stall metabolic processes (Almeida *et al*., 2007; Almeida *et al*., 2009; Hasunuma & Kondo, 2012; Keating *et al*., 2006). MF15 and MY5 on the other hand, performed well in the presence of the synthetic inhibitor cocktail; these two strains can therefore be considered as inhibitor tolerant, because they had the ability to ferment regardless of the presence of inhibitors.

4.5 Ethanol tolerance

None of the parental or hybrid strains had the ability to ferment glucose in the presence of 10% ethanol (Figure 25 and 26). The lack of glucose fermentation could be due to the fact that ethanol inhibits the glucose transport systems and disrupts the cell membrane structure. The strains could also not produce any ethanol throughout the two-week period. This might be attributed to the initial ethanol concentration being too high that might have disturbed the homeostasis of the cell membrane to H^+, which led to intracellular acidification and degradation of ATP by the ATPase due to an influx of H^+ into the cell (Quintas *et al*., 2000). This could ultimately have led to the inability of the strains to ferment. A slight decrease in the ethanol concentration over time was observed and could be due to the evaporation of the ethanol during the preparation of the HPLC samples or during the fermentation at 30°C.

The p-values for both fermentation sets in terms of glucose consumption and ethanol production was greater than 0.05, i.e. no significant differences were observed between the strains. The means for residual glucose and ethanol concentration (Table 6) also reveal no differences as all of the strains' mean averages were in the same range.

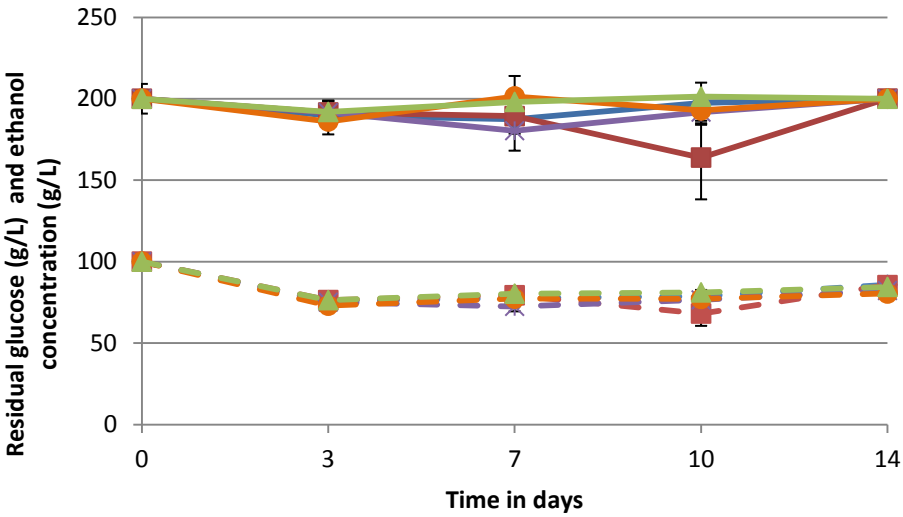

Figure 25: Residual glucose (—) and ethanol concentration (--) by *S. cerevisiae* strains HR14 (—◆—) and YI64 (—■—) with hybrid YH1 (—✳—), YH2 (—●—) and YH3 (—▲—) strains in MNS medium + 10% ethanol.

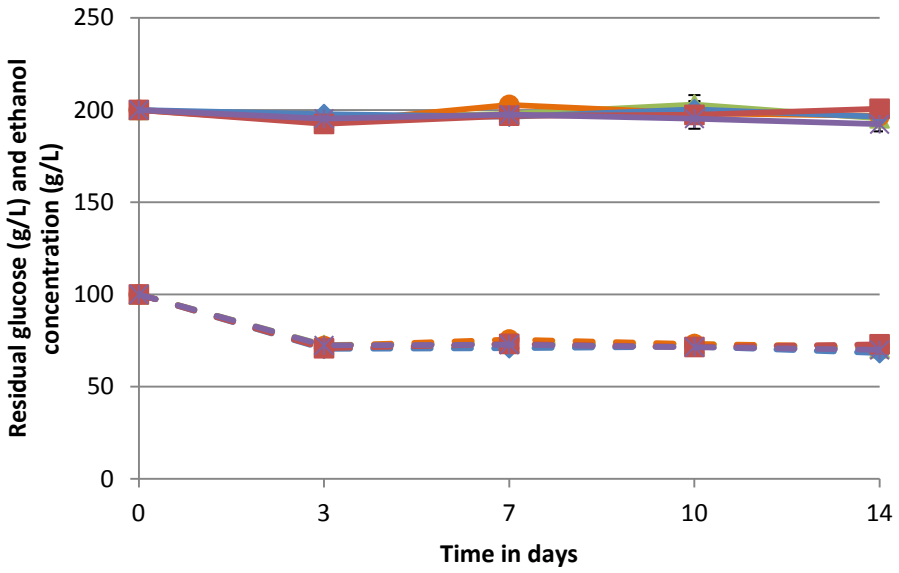

Figure 26: Residual glucose (━) and ethanol concentration (--) by *S. cerevisiae* strains MF15 ( ) and YI2 ( ) with hybrid MY3 ( ), MY5 ( ) and MY7 ( ) strains in MNS medium + 10% ethanol.

Table 6: Mean residual glucose (g/L) and ethanol concentration (g/L) by yeast strains over fourteen days

Strains	N	Glucose	Ethanol	Strains	N	Glucose	Ethanol
HR14	15	197.38a	83.573a	MF15	15	199.172a	76.243a
YI64	15	190.58a	81.887a	YI2	15	197.443a	77.701a
YH1	15	194.97a	81.281a	MY3	15	196.045a	77.295a
YH2	15	196.51a	81.565a	MY5	15	198.184a	78.475a
YH3	15	201.05a	84.465a	MY7	15	198.390a	78.044a
LSD (p=0.05)		22.79	3.4249	LSD (p=0.05)		7.1088	2.459

Values in the same column followed by the same letter indicate no significant difference. Least Significant Difference (LSD). N refers to the number of replicates multiplied by the total readings taken for each strain.

4.6 Growth characteristics

The parental HR14 and YI64 strains, together with their hybrid progeny YH1, YH2 and YH3, grew well in standard MNS medium (Figure 27 A). The growth curve followed the normal progression in batch culture. No or a minimal lag phase, stationary phase between day 2 and 5 (YI64 and YH2). The curve then shows a decline at day 5 as the cells began to die off. However, all the strains struggled to grow in the presence of either the 25% synthetic inhibitor cocktail (Figure 27 B) or 10% ethanol (Figure 27 C). These results correlate with the poor fermentation performance of all of the strains under these inhibitory conditions.

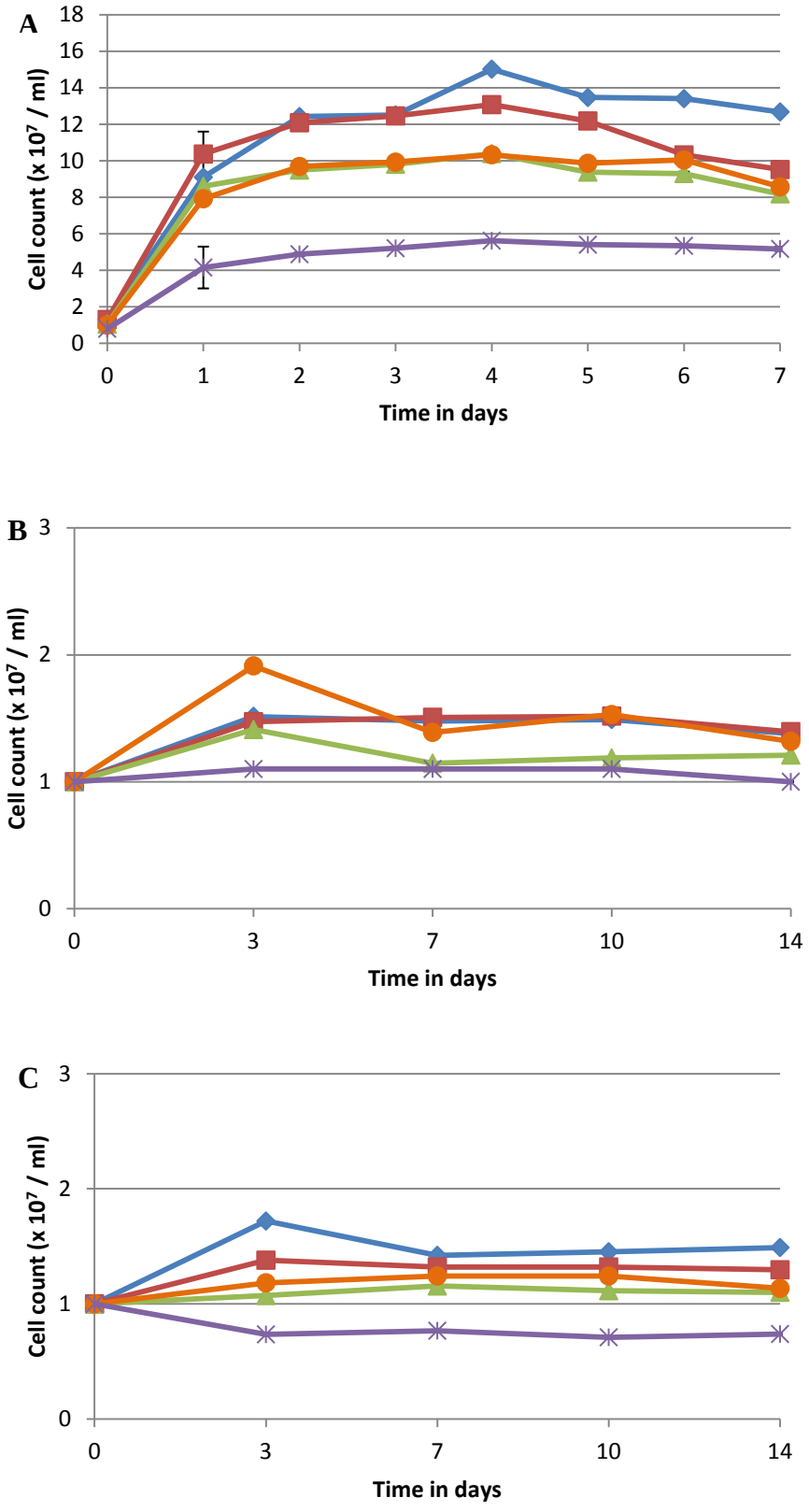

Figure 27: Growth curves of parental *S. cerevisiae* strains, HR14 ( ) and YI64 ( ) and hybrids, YH1 ( ), YH2 (and YH3 ( ) in (A) MNS medium, (B) MNS + 25% inhibitor cocktail, or (C) MNS + 10% ethanol.

The MF15, YI2 parental strains and their hybrid strains also followed a normal growth progression pattern (Figure 28 A). Unlike the HR14 and YI64 strains, the MF15 and YI2 parental and hybrid strains could grow in the presence of 25% inhibitor cocktail (Figure 28 B), although some variation in the growth patterns was evident. The MF15 strain followed a normal growth progression with stationary phase reached at day 7, whereas the rest of the strains grew slower and reached stationary phase only at day 10. These results correlate with the fermentation performance of these strains during the 14 day period. The results in Figure 28 C indicate that the parental YI2 parental and hybrid MY3, MY5 and MY7 strains could grow to a limited extend in the presence of 10% ethanol, whereas the MF15 parental strain grew considerably better in the presence of 10% ethanol than the other strains.

The results indicated that all the strains grew well under non-selective conditions, but as soon as pressure (25% inhibitors or 10% ethanol) was introduced, the HR14 and YI64 parental and hybrid strains struggled to grow. It can be concluded that these strains are neither inhibitor nor ethanol tolerant as they were unable to grow or ferment in the presence of inhibitors or ethanol. In contrast, the MF15 and YI2 parental and hybrids strains were able to grow in the presence of 25% inhibitor cocktail yielding similar cell counts as in standard MNS (Figure 28 B). MF15 displayed a higher inhibitor tolerance than YI2 as indicated in Figure 28 B. They did, however, struggle to grow in the presence of 10% ethanol and could therefore be regarded as inhibitor tolerant, but not ethanol tolerant. However, the MF15 parental strain was able to grow better than the other strains in the presence of both inhibitors and ethanol.

Strain HR14 was initially characterized as an inhibitor tolerant strain, but in this fermentation growth trial this characteristic was not displayed as this strain struggled to grow in the presence of 25% inhibitor cocktail. Strain YI64 on the other hand initially displayed a good fermentation vigour (consumed glucose fast) characteristic and this was confirmed in the fermentation growth trial without selective pressure (standard MNS medium). Strain MF15 was characterized as an osmotolerant yeast and the results in the three separate fermentation growth trials confirm that this yeast strain could grow in the presence of high glucose concentrations. Strain YI2 displayed an ethanol tolerant characteristic, but the growth (in presence of 10% ethanol) result does not correlate with this characteristic, because this strain struggled to grow in the presence of 10% ethanol in the growth medium. Strain MF15 on the other hand was able to grow in the presence of 10% ethanol although it was not initially characterize as an ethanol tolerant yeast. The growth results agree in some instances with the previous screening characterisation and in some instances show unexpected results.

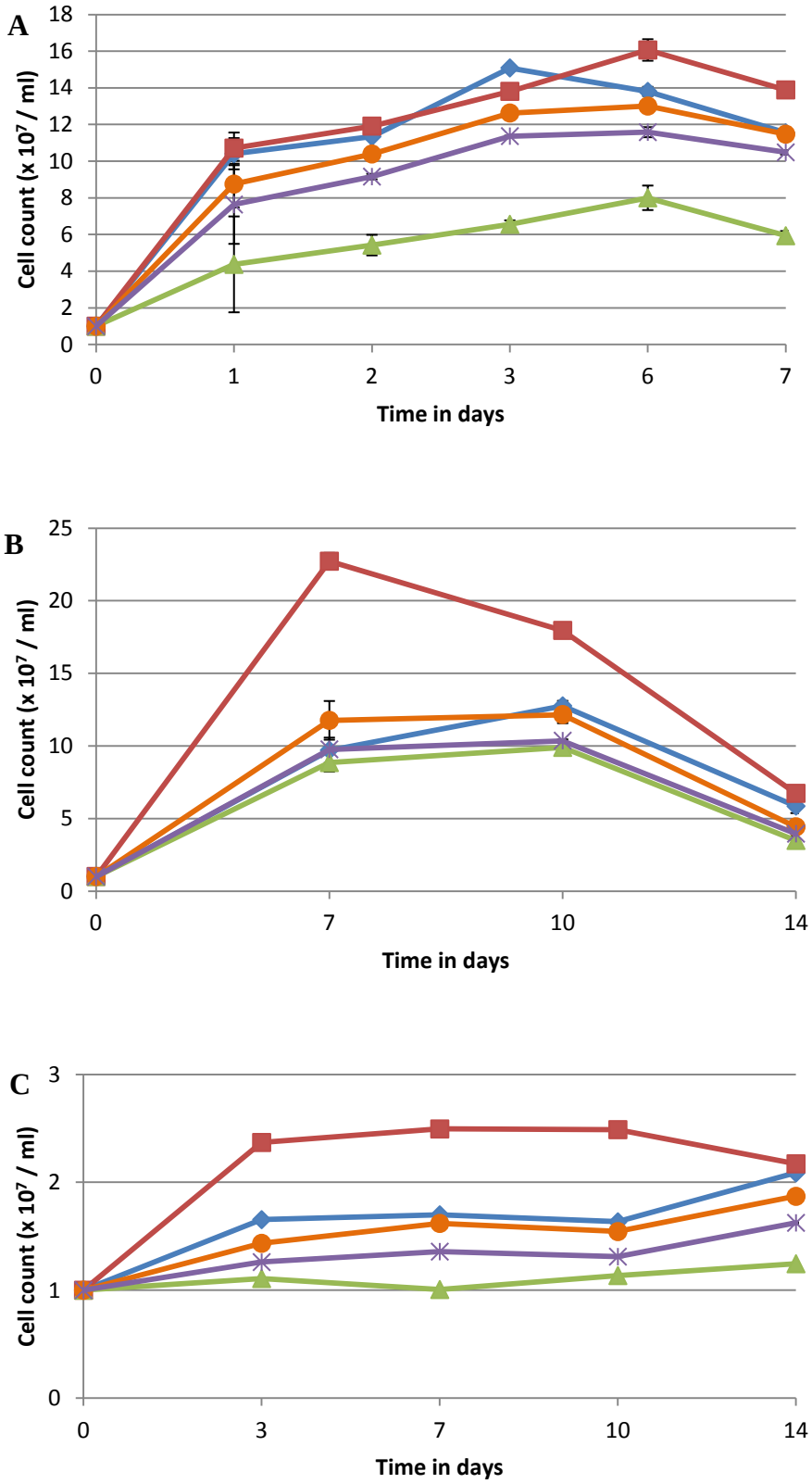

Figure 28: Growth curves of parental *S. cerevisiae* strains, YI2 ( ) and MF15 ( ) and hybrids, MY3 ( ), MY5 ( ) and MY7 ( ) in (A) MNS medium, (B) MNS + 25% inhibitor cocktail, or (C) MNS + 10% ethanol.

4.7 Hybrid stability

The best performing hybrid strains, YH3 and MY5, grew well after each of the 10 consecutive transfers. They were able to retain the disruption cassettes and the insert was stable for a number of generations.

4.8 Ethanol adaptation

The HR14, YI64 and YI2 parental strains, grew slower than the MF15 parental strain in MNS medium supplemented with 10% ethanol. The adapted and unadapted hybrids of YH3 and MY5 grew much faster than the three above-mentioned parental strains (Figure 29). The growth curve followed the normal progression and the stationary phase was reached on day 4. For the YI2 parental strain the stationary phase was already reached at day 3. The results indicate that adaptation has conferred the hybrid strains with slightly greater tolerance to 10% ethanol since they were capable of better growth compared to the three parental strains.

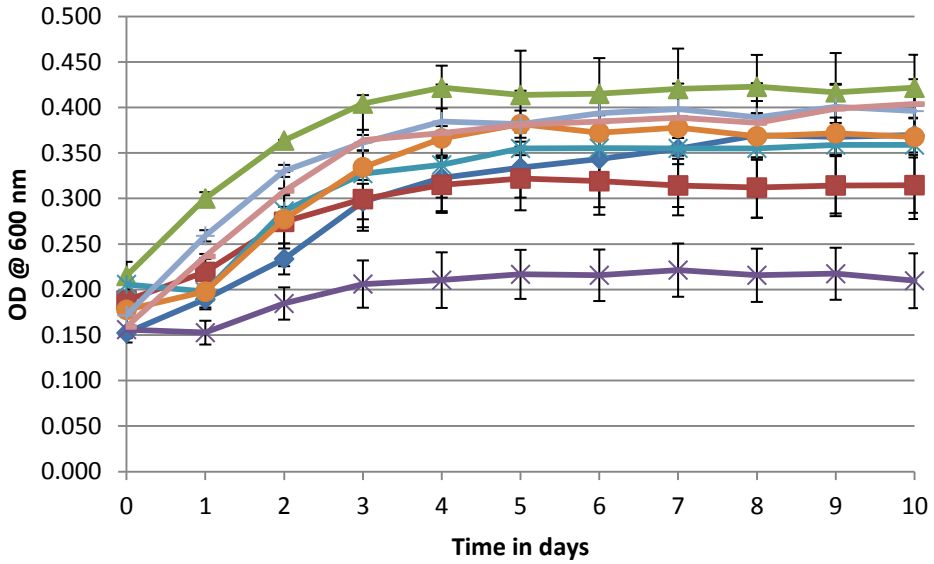

Figure 29: Growth curve of the parental *S. cerevisiae* strains, HR14 ( ), YI64 ( ), MF15 ( ) and YI2 ( ) adapted hybrids YH3 ( ) and MY5 ( ) as well as unadapted hybrids YH3 ( ) and MY5 ( ) in MNS medium supplemented with 10% ethanol.

The MF15 parental strain displayed tolerance to ethanol during this screening experiment. This parental strain grew slightly better than the hybrid strains in the presence of 10%

ethanol. It can be concluded that adaptation rendered the MF15 parental strain as well as the YH3 and MY5 hybrid strains more tolerant to high ethanol concentrations.

There were no striking differences observed in the morphology of the unadapted and adapted strains when grown under selective pressure (10% ethanol) over the 10-day period (Figure 30 and 31). However, the cells of the MY5 adapted strain were slightly bigger than those of the unadapted strain (panels C & D in Figures 30 and 31), and the YH3 hybrid strain showed signs of flocculation (panels A & B in Figures 30 and 31). The morphology of YH3 and MY5 hybrid strains differed as indicated in the results. These two hybrids also differed with regard to the colony morphology on YPD agar plates (results not shown): StrainYH3 hybrid colonies appeared irregular with a rough texture, whereas MY5 displayed a smooth edge and texture.

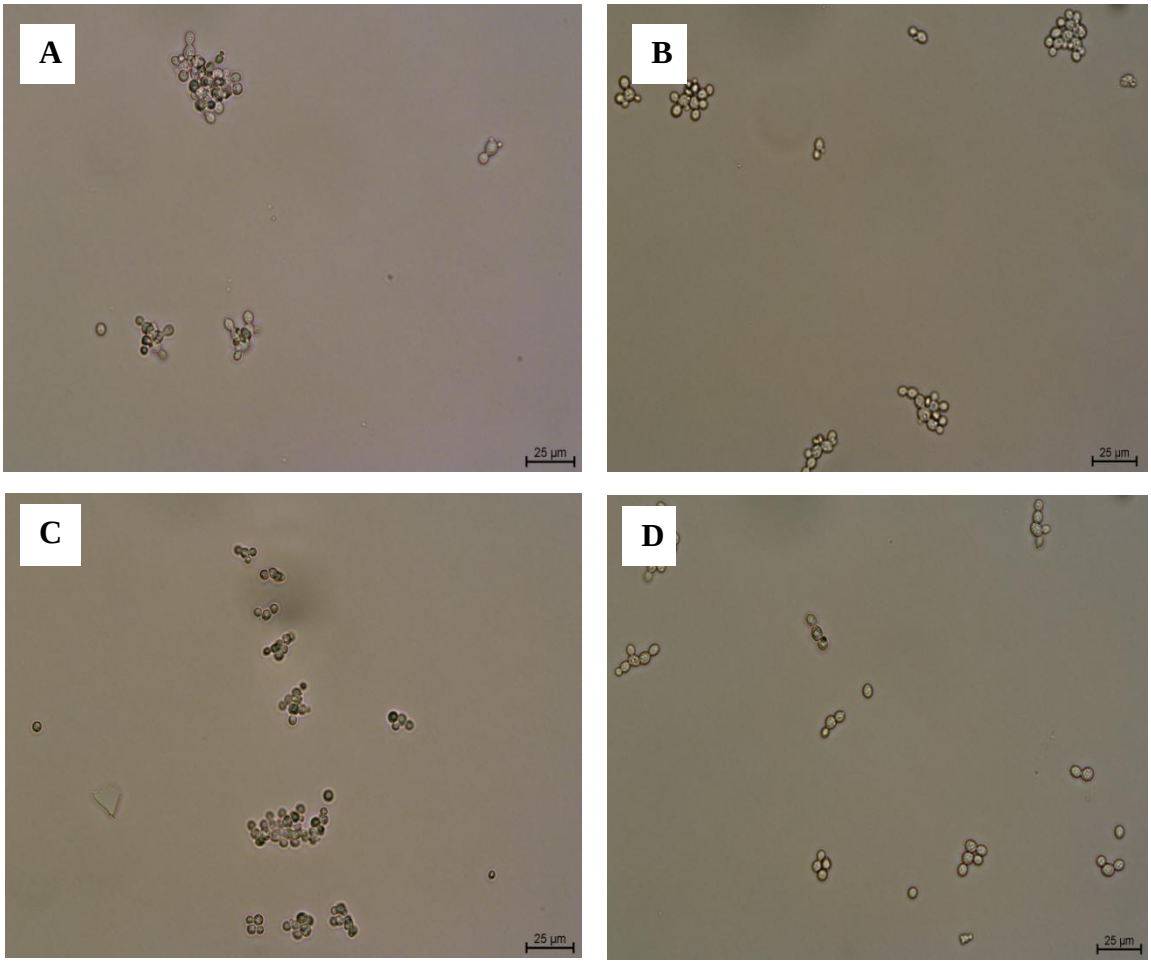

Figure 30: Morphology of the unadapted (A) YH3 and (C) MY5 strains and the adapted (B) YH3 and (D) MY5 strains on day 1 of cultivation in MNS medium containing 10% ethanol.

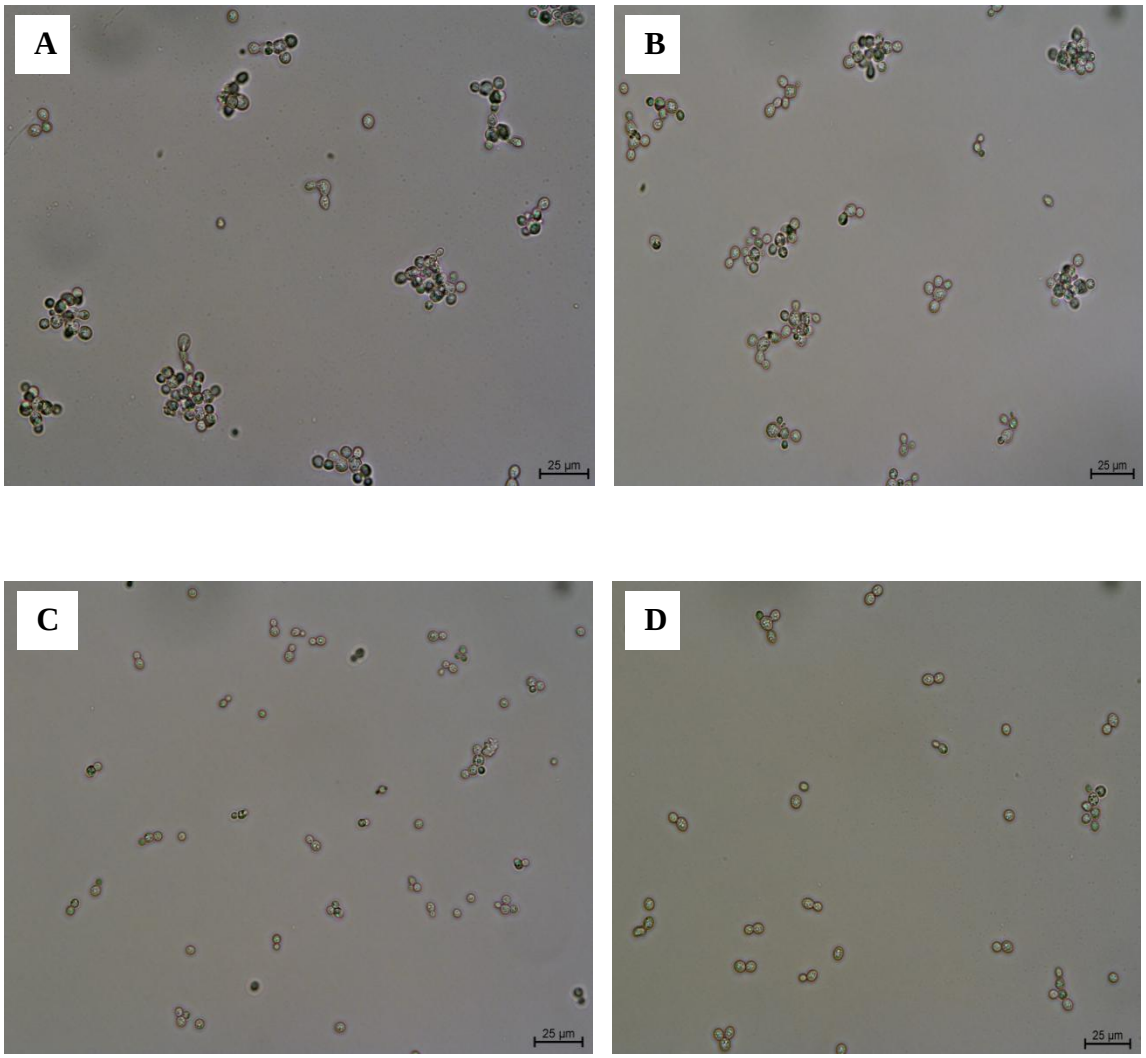

Figure 31: Morphology of the unadapted (A) YH3 and (C) MY5 strains and the adapted (B) YH3 and (D) MY5 strains on day 10 of cultivation in MNS medium containing 10% ethanol.

4.9 Fermentation studies on adapted strains

Both the adapted and unadapted YH3 hybrid strains displayed poor fermentation abilities in the presence of 10% ethanol (Figure 32 A), similar to the HR14 and YI64 parental strains. There was a slight decrease of the initial 200 g glucose over the 14 day period for most of the strains, except for the YH3 unadapted (no glucose was utilised) and HR14 (clear decrease in glucose concentration) strains. A slight increase in the ethanol concentration was observed for all of the strains, which produced ± 25 g/L ethanol in addition to the initial 10%. The low fermentation ability and low ethanol production of all the strains could be attributed to the

fact that ethanol inhibits the glucose transport system of the cells and disrupts the cell's membrane structure (Ansanay-Galeote *et al*., 2001; Hu *et al*., 2007).

Ethanol adaptation did not improve the strains' ability to grow or ferment in the presence of 10% ethanol. The p-values for both glucose consumption and ethanol production were less than 0.05, indicating that there was a significant difference between the data generated by the strains during this fermentation trial. The mean glucose and ethanol values (Table 7) for most of the strains were in the same range, except for HR14, which had better glucose consumption. This strain did, however group with the YI64 parent strain with respect to ethanol production. The adapted as well as the unadapted YH3 strains displayed similar mean glucose and ethanol values.

The MF15 and YI2 parental strains as well as the adapted and unadapted of MY5 strains also displayed poor fermentation ability in the presence of 10% ethanol (Figure 32 B). The MF15 and unadapted MY5 strains did not utilise glucose, but managed to produce some ethanol during the two-week period. There was a fluctuation in the glucose consumption by YI2 and adapted MY5 and their ethanol production was also low. An average of 25 g/L (excluding the initial 10%) ethanol was produced by all the strains after 14 days. As explained above, the initial ethanol might have had a toxic effect on these cells that led to them to employ measures to detoxify the ethanol and the cells therefore entered an extended lag phase to rectify the damage. The p-values for this fermentation trial were less than 0.05 for glucose consumption, but greater than 0.05 for ethanol production. This implies that there was a significant difference in the data for glucose consumption, but not for ethanol production. This result is also confirmed by the glucose consumption and ethanol production means (Table 7) where the data produced for glucose consumption by the YI2 parental strain differed significantly from the other three strains (i.e. consumed its glucose faster).

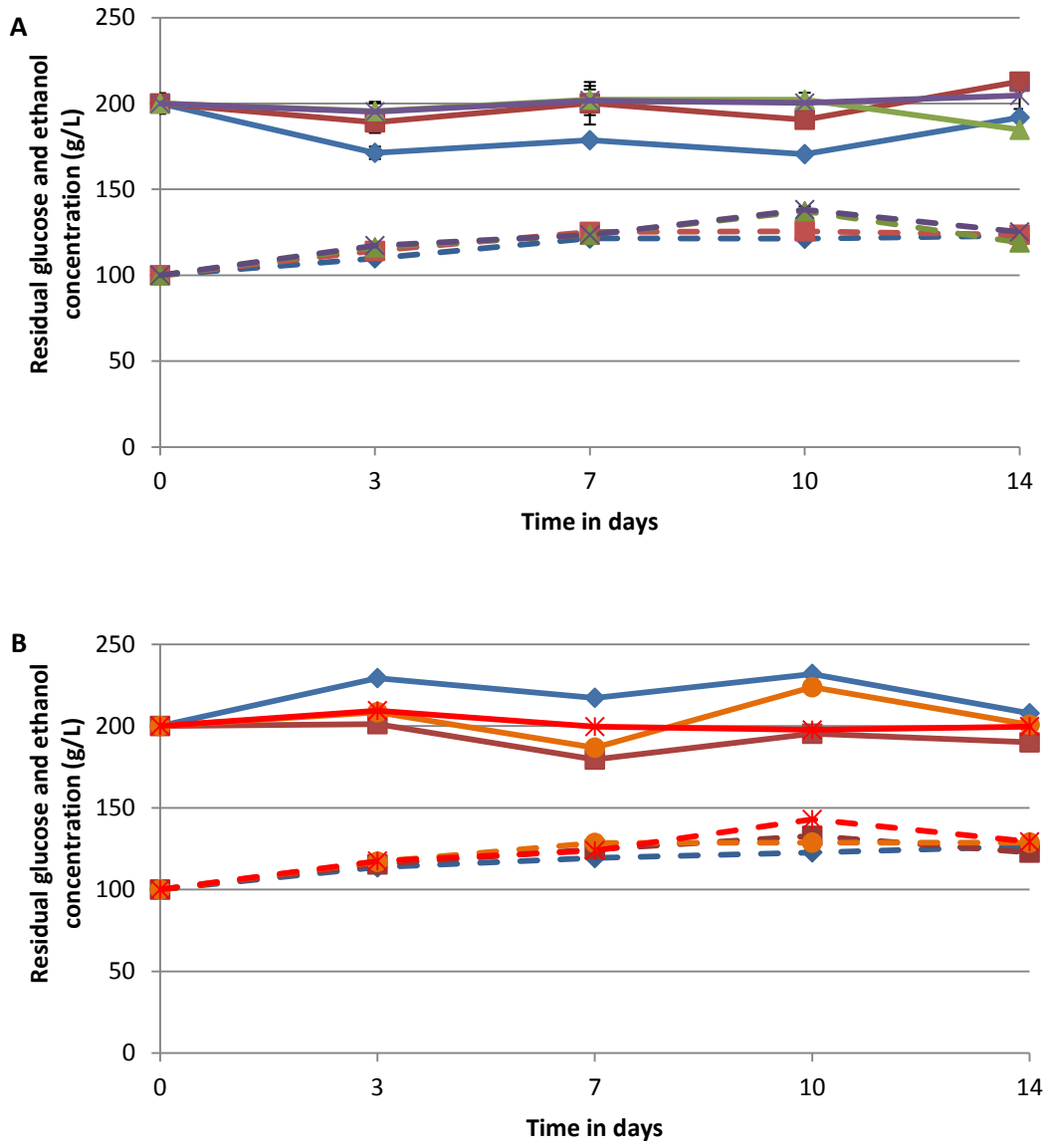

Figure 32: (A) Residual glucose (—) and ethanol concentration (--) by *S. cerevisiae* strains HR14 ( ) and YI64 ( ) with adapted hybrid YH3 ( ) and unadapted hybrid YH3 ( ). (B) *S. cerevisiae* strains MF15 ( ) and YI2 ( ) with adapted hybrid MY5 ( ) and unadapted hybrid MY5 ( ).

Table 7: Mean residual glucose (g/L) and ethanol concentration (g/L) by yeast strains over fourteen days

Strains	N	Glucose	Ethanol	Strains	N	Glucose	Ethanol
HR14	15	182.456b	115.105b	MF15	15	206.824a	116.410b
YI64	15	198.481a	117.708ab	YI2	15	182.763b	119.090ab
YH3 adapted	15	196.943a	119.240a	MY5 adapted	15	196.078a	120.575ab
YH3 unadapted	15	200.384a	120.868a	MY5 unadapted	15	198.733a	122.685a
LSD (p=0.05)		12.542	3.8964	LSD (p=0.05)		12.36	4.5203

Values in the same column followed by the same letter indicate no significant difference. Least Significant Difference (LSD). N refers to the number of replicates multiplied by the total readings taken for each strain.

None of the strains reported in Figure 32 could be regarded as ethanol tolerant as they showed poor fermentation abilities and produced low concentrations of ethanol. There was a slight decrease in the glucose concentration for most of the strains, except for the unadapted YH3 that showed almost no glucose consumption. The ANOVA test results support these findings and confirmed that there was a significant difference in the generated data as the p-values were less than 0.05. The poor glucose utilisation also corresponds to the low ethanol production. It seems that the initial presence of ethanol had an inhibitory effect on the fermentation performance of these strains.

Although the MF15 and unadapted MY5 strains could not effectively utilise glucose, they managed to produce an additional 15 g/L ethanol (Figure 32 B). The YI2 and adapted MY5 strains utilised some of their glucose and also produced an average of 25 g/L ethanol. These strains could also not be regarded as ethanol tolerant as they struggled to ferment in the presence of 10% ethanol. The ANOVA results indicate that the strains differed significantly in their ability to consume glucose, but that differences in ethanol production were not significant.

4.10 Fermentation studies with triticale straw hydrolysate

The YI64 parental and unadapted YH3 strain performed well on triticale hydrolysate; they utilised all the glucose within 7 days and produced approximately 15 g/L ethanol by day 14 (Figure 33 A). The adapted YH3 strain took 14 days to utilise all its glucose and also produced approximately 15 g/L ethanol by day 14. This result confirmed that the adaptation did not improve the fermentation ability of the YH3 hybrid strain, since the unadapted strain performed much better in terms of glucose consumption. The HR14 parental strain was unable to utilise the glucose throughout the two-week period and only started to produce

ethanol from day 10. This strain seem to have entered an extended lag phase that might be due to the presence of the inhibitors, acetic acid, formic acid, FF and HMF in the triticale hydrolysate. The HR14 strain may be regarded as intolerant to inhibitors, whereas the rest are inhibitor tolerant and good fermenters.

The p-value for this fermentation assessment was less than 0.05, which indicate that the results were significant. The t-Test (Table 8) confirms that there were differences between the means of the glucose consumption and ethanol production. The YI64 parental strain and the unadapted YH3 hybrid strain produced significantly higher amounts of ethanol in comparison to the other two strains. Strains YI64 and the unadapted YH3 also performed similarly with regard to both glucose consumption. The ANOVA and t-Test also confirms that the adaptation process has no impact on the strain's performance as the unadapted YH3 perform better than the adapted YH3 in both glucose consumption and ethanol production.

Table 8: Mean residual glucose (g/L) and ethanol yields (g/L) by yeast strains over fourteen days

Strains	N	Glucose	Ethanol	Strains	N	Glucose	Ethanol
HR14	15	26.473a	1.9247c	MF15	15	17.7393a	4.110b
YI64	15	7.149c	9.4760a	YI2	15	7.0120c	9.991a
YH3 adapted	15	15.447b	6.2460b	MY5 adapted	15	13.9407b	6.513ab
YH3 unadapted	15	9.668c	8.9380a	MY5 unadapted	15	19.1473a	5.182b
LSD (p=0.05)		4.2451	2.0662	LSD (p=0.05)		1.8874	3.7936

Values in the same column followed by the same letter indicate no significant difference. Least Significant Difference (LSD). N refers to the number of replicates multiplied by the total readings taken for each strain.

The YI2 parental strain performed better than the MF15 parental strain and adapted and unadapted MY5 strains (Figure 33 B). It consumed all the residual glucose by day 7 and produced approximately 15 g/L ethanol by day 14. MF15 and the other two strains only started to utilise glucose on day 7 and produced varying amounts of ethanol. MF15 produced approximately 10 g/L ethanol by day 14, whereas the adapted and unadapted MY5 strains produced approximately 15 g/L and 6 g/L, respectively, on day 14. It seem that the adaptation might have improved the hybrid MY5's performance as it performed much better than the unadapted strain, both in terms of glucose utilisation and ethanol production. The ANOVA test confirmed that there were significant differences between the data. A significant difference is evident for YI2 for the means of both the glucose consumption and ethanol production (Table 8).

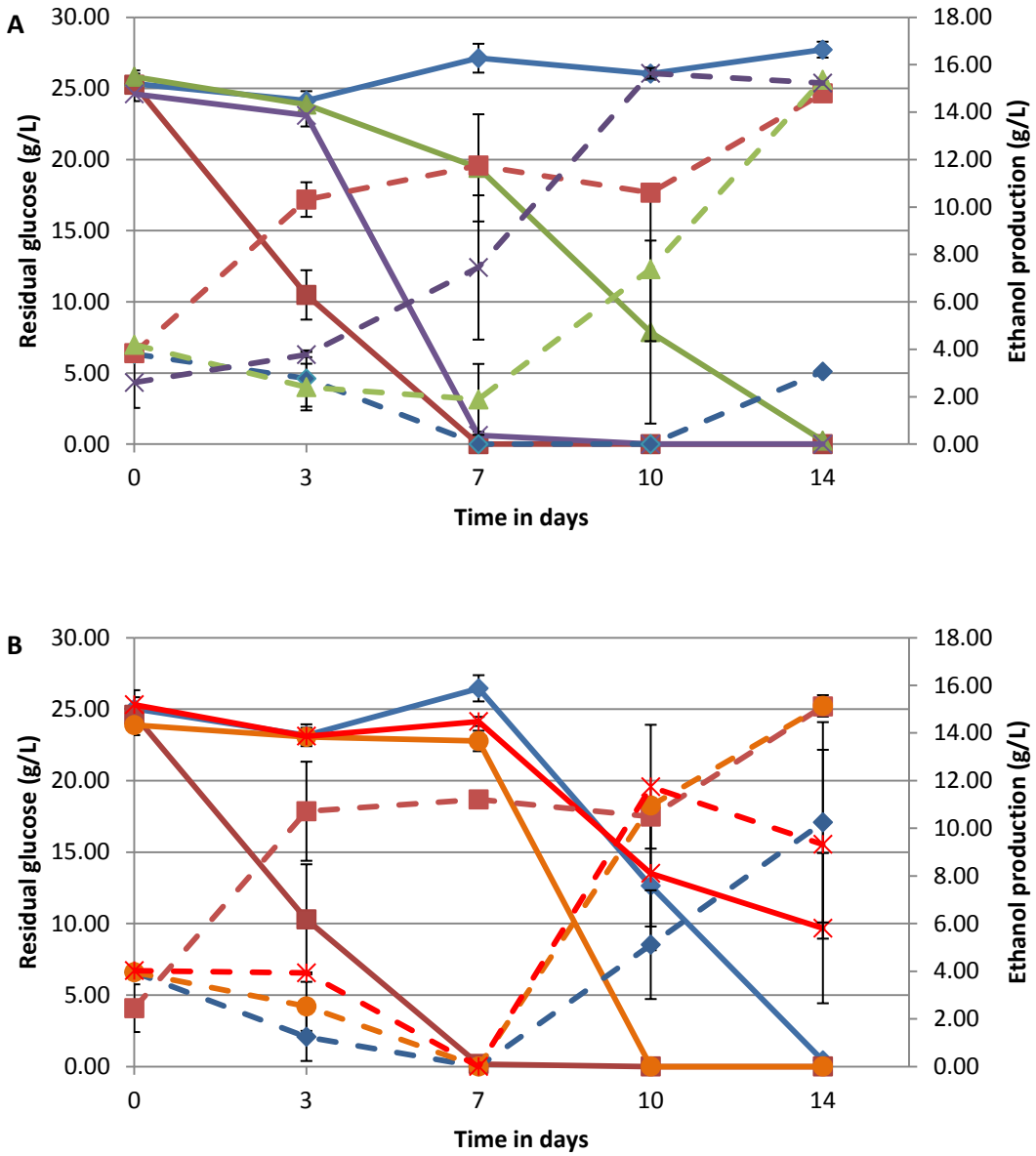

Figure 33: (A) Residual glucose (—) and ethanol production (--) by *S. cerevisiae* strains HR14 ( ) and YI64 ( ) and adapted hybrid YH3 ( ) and unadapted hybrid YH3 ( ) cultivated on triticale straw hydrolysate. (B) *S. cerevisiae* strains MF15 ( ) and YI2 ( ) and adapted hybrid MY5 ( ) and unadapted hybrid MY5 ( ) cultivated on triticale straw hydrolysate.

Most of the strains seem to have good fermentation abilities except for the HR14 parental strain (Figure 32 A and B). They were able to utilise the available glucose and produce some ethanol during the two-week period. The adaptation seems to have had no effect on the development of YH3 as the unadapted strain performed better than the adapted one.

The inhibitors within the triticale hydrolysate did not seem to have a negative effect on most of the strains' fermentation performances, suggesting that they could be regarded as inhibitor tolerant strains. The adaptation seems to have had a positive effect on the MY5 hybrid, which performed better than the unadapted strains in terms of glucose consumption during the fermentation trial (Figure 33 B). Most of the strains (except for HR14) seem to have the ability to utilise a lignocellulosic substrate such as triticale straw. The two parental strains, YI64 and YI2, as well as the unadapted YH3 and adapted MY5 hybrid strains were able to ferment the triticale straw hydrolysate. These strains had the ability to withstand the negative effects of the inhibitors present in the triticale straw hydrolysate and can therefore be regarded as inhibitor tolerant strains. The hybrids were, however, unable to perform better than their parental strains on triticale straw hydrolysate; in fact, one parent of each set, i.e. YI64 and YI2 outperformed the hybrids.

4.11 Thermotolerance

The adapted and unadapted hybrid strains of YH3 and MY5 had the ability to grow at 40, 42 and 43°C, but struggled to grow at 44°C. The corresponding parental strains were able to grow at a maximum temperature of 40°C (Mrs. Trudy Jansen), with the YI64 parental strain able to grow at 43°C. The hybrid strains were able to grow at 43°C (Figure 34). It is therefore noteworthy that the hybrids managed to grow well at 43°C, which makes them slightly more tolerant to higher temperatures than their parental counterparts. Since *S. cerevisiae* strains are known to optimally grow at 25 – 30°C (Kawamura, 1999), this implies that the hybrid strains exceeded the known temperature boundaries and can be viewed as thermotolerant yeasts. The mating experiments therefore yielded positive results with regard to the temperature tolerance of the hybrid strains as they were able to grow at 43°C.

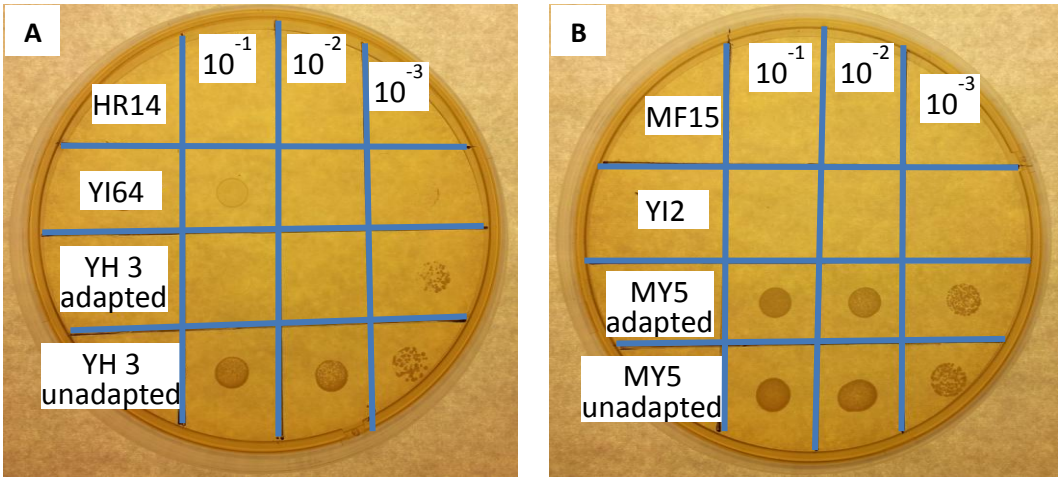

Figure 34: Growth of *S. cerevisiae* strains HR14, YI64, adapted and unadapted YH3 (A) and MF15, YI2 and the adapted and unadapted MY5 (B) at 43°C.

4.12 Osmotolerance

Slight differences in growth was observed for the adapted and unadapted YH3 strains when compared to the parental HR14 and YI64 strains in the presence of 60% glucose, with the hybrid strains that grew slightly better than the parental strains (Figure 35 A). However, no real difference in growth could be observed for the parental strains and adapted YH3 strain in the presence of 65% glucose (Figure 35 B), whereas the unadapted YH3 hybrid grew slightly weaker than the other strains. The adapted and unadapted MY5 strains grew better than the parental YI2 strain in the presence of 60 and 65% glucose (Figure 35 C, D), whereas their growth corresponds with the MF15 parental strain and confirms the initial finding that MF15 is osmotolerant. The adapted and unadapted hybrid strains grew slightly better than the parental strains in the presence of 60% glucose as indicated in Figure 35 A. Unadapted YH3 grew more weakly in the presence of 65% glucose than the other strains (Figure 35 B). Despite the slightly lower growth of the unadapted YH3 in the presence of 65% glucose, the hybrid strains were able to tolerate high concentrations of glucose in their growth media. Together with the parental strains, the hybrid strains may be regarded as osmotolerant. In the initial screening done by Mrs. Jansen (unpublished), HR14 was unable to grow in the presence of 60% glucose and thus not regarded as osmotolerant, whereas YI64 was able to grow in the presence of 60% glucose and regarded as an osmotolerant yeast. It can be noted that in this screening experiment HR14 managed to grow in the presence of 60% glucose and can therefore be viewed as an osmotolerant yeast. YH3, which is the progeny of HR14 and YI64, seems to have inherited the osmotolerant characteristic from YI64.

This screening process confirms the initial finding that MF15 is osmotolerant. Both the adapted and unadapted MY5 hybrid strains grew slightly better than the parental MF15 strain and could be regarded as osmotolerant yeasts. YI2 did not grow as well as the other yeasts in the presence of high glucose concentrations, which is in agreement with the initial finding (Trudy Jansen, unpublished) that strain YI2 was not osmotolerant. The osmotolerant trait of MY5 was therefore inherited from the parental MF15 strain. The mating experiment managed to reach its goal in this regard as the hybrid MY5 strain displayed a slightly superior osmotolerance that the parental YI2 strain failed to display. Growth of MY5 was also slightly better than that of MF15.

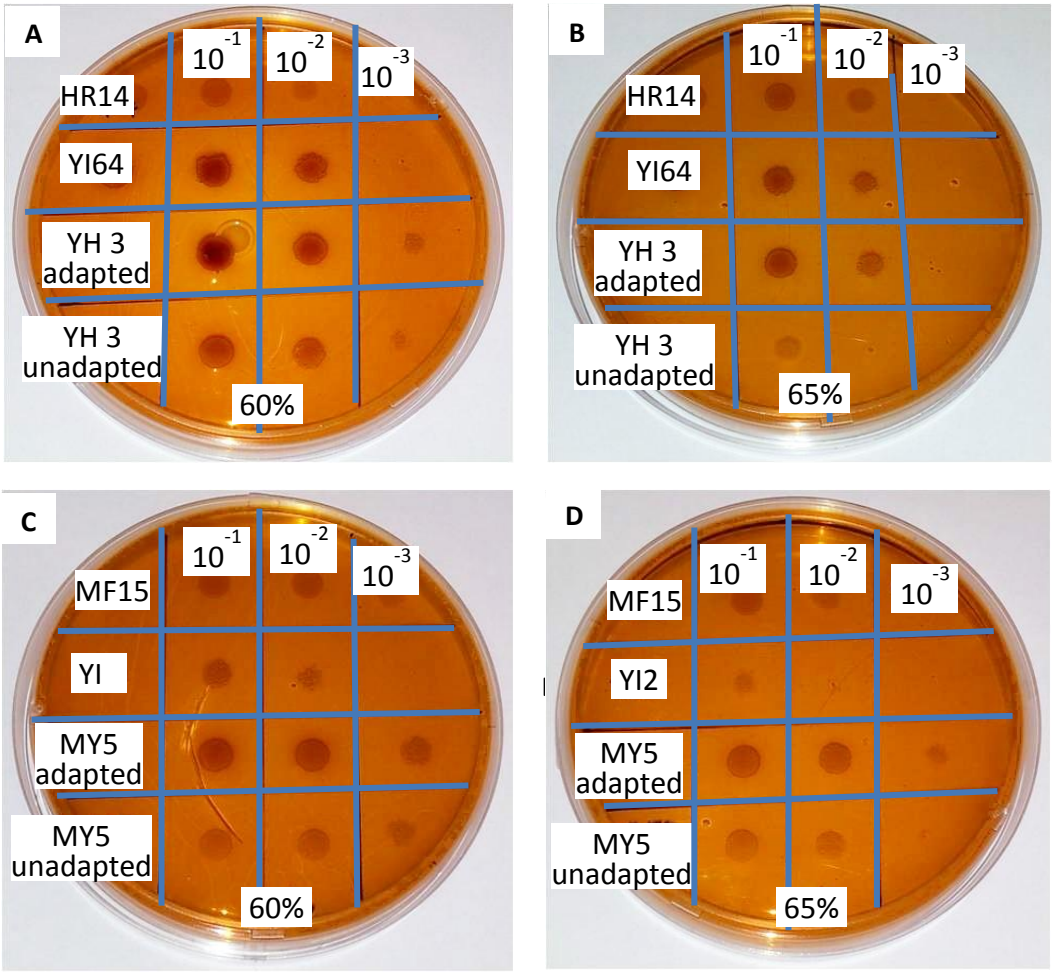

Figure 35: Growth of *S. cerevisiae* strains HR14, YI64, adapted and unadapted YH3 in the presence of (A) 60 and (B) 65% glucose, respectively. Growth of *S. cerevisiae* strains MF15, YI2, adapted and unadapted MY5 in the presence of (C) 60 and (D) 65% glucose, respectively.

Chapter 5: General summary and conclusion

The transport sector and the world's economy rely heavily on fuel. A shortage thereof will drastically alter modern life, as we know it. The production of bio-ethanol is pursued due to the environmental concern with regard to global warming and the rise in the earth's temperature, the insecurity of the availability of fossil fuels and the resulting high fuel prices (Balat, 2011; Dias *et al.*, 2009; Gasparatos *et al*., 2011). In Brazil and the USA, bio-ethanol is mainly produced from feedstock such as sugarcane bagasse and corn crops (Azadi *et al*., 2012; Kasavi *et al*., 2012; Searchinger *et al*., 2008).

Bio-ethanol can be used as a substitute for petroleum. It can be produced from a variety of sources that include wood, compost, household waste and agricultural waste products (Fujitomi *et al*., 2012; Gasparatos *et al*., 2011; Hughes *et al*., 2009). Bio-ethanol production is based on the ability of *S. cerevisiae* to ferment the sugars to ethanol. A robust *S. cerevisiae* strain with all the desired characteristics such as fermentation vigour, ethanol tolerance, inhibitor tolerance, osmotolerance and temperature tolerance is highly sought after (Benjaphokee *et al*., 2012; den Haan *et al*., 2013; den Haan *et al*., 2015; Fujitomi *et al*., 2012; Garay-Arroyo *et al*., 2004; Hahn-Hägerdal *et al*., 2001). Such a strain could be generated through crossbreeding strategies using strains that display the above-mentioned characteristics. Crossbreeding is a method commonly used in the wine industry to obtain hybrid progeny with combined and improved oenological characteristics (Pérez-Través *et al*., 2012; Pretorius, 2000; Rainieri & Pretorius, 2000). Based on this principal and the successful outcome that the wine industry has experienced, this method was used in this study.

The *S. cerevisiae* isolates were obtained from the culture collection of the ARC Infruitec - Nietvoorbij and were screened for their ability to ferment sugars, withstand harsh temperatures, ethanol tolerance, osmotolerance and inhibitor tolerance. Four isolates (HR14, YI64, YI2 and MF15) displaying different characteristics were selected for this study. Neither of these strains possessed all of the required characteristics for a robust strain. Therefore, a mating strategy was employed to obtain a superior strain. Natural strains of *S. cerevisiae* normally display a functional *HO* gene (homothallism) that is responsible for the mating-type switching that allows spores of the same parent to mate and generate diploid progeny. The *HO* gene of the diploid homothallic yeast strains was disrupted through targeted deletion-disruption strategies to produce haploid heterothallic strains. Haploid strains with the opposite mating-types and displaying different characteristics were mated to produce hybrid

strains with combined / superior characteristics. Several hybrid strains were obtained and three from each parental set were selected and screened for their fermentation abilities. The best performing hybrid strains, YH3 and MY5 was further adapted to 10% ethanol to enhance their fermentation performance. These two hybrid strains along with the parental strains were screened for their osmotolerance and ability to grow at temperatures exceeding 30°C.

The adaptation had a positive effect on the MY5 hybrid as the adapted strain was able to utilise glucose faster than the unadapted strain during the fermentation trial on triticale hydrolysate. The adaptation did not have any effect on the YH3 hybrid as the unadapted strain performed better during fermentation. The adapted and unadapted *S. cerevisiae* YH3 and MY5 hybrid strains had the ability to grow at 43°C in contrast to the HR14, YI2 and MF15 parental strains. The YI64 parental strain could grow at 43°C, but grew more weakly than the hybrid strains. Although the hybrid strains had the ability to grow at 43°C, their ability to ferment at this temperature still needs to be evaluated. Furthermore, their overall fermentation ability (vigour) was not superior to that of the parent strains.

Most of the desired characteristics such as good fermentation abilities, inhibitor tolerance, osmotolerance and thermotolerance were displayed by the YH3 and MY5 hybrids. Ethanol tolerance only became evident for the hybrid strains after adaptation. Some of these characteristics displayed by the hybrids were not superior to that of the parent strains. For example, YI64 and YI2 performed similarly to the YH3 and MY5 hybrid strains, during separate fermentation trails. These two parent strains were regarded as good fermenters and their hybrid strains performed on par with them. The YI2 parent strain, was initially characterised as an ethanol tolerant strain, but did not display this characteristic during this fermentation assessment. None of the parent or the hybrid strains had the ability to ferment in the presence of ethanol and cannot be regarded as ethanol tolerant strains. The MF15 parent strain is an inhibitor tolerant strain and its MY5 hybrid inherited this trait. This parent strain performed notably better than its hybrid in the fermentation trails.

The mating experiments resulted in the generation of *S. cerevisiae* hybrid strains with multiple traits, but these traits were not enhanced, as the hybrid strains in most instances did not perform better than the parent strains. There were, however, instances where the hybrid strains performed notably better than the parent strains. These include slightly higher osmotolerance than some parent strains and better growth at 43°C than the parent strains. This study therefore resulted in the generation of hybrids with combined traits, but not

superior traits. To enhance these traits additional adaptation trails could be used that might improve these individual traits. By virtue of this outcome and in light of the result of this study, the classical mating strategy is still a recommended method for the combining of several characteristics of different parental strains in a single progeny.

Future research could involve conducting a breeding selection programme (mating experiments) where the hybrid strains generated in this study could be used as starting material or parent strains for the programme. Subsequent mating experiments could be conducted until hybrid progeny with the desired combined and superior characteristics are obtained. In these mating experiments hybrids are produced through minimum gene manipulation. These hybrids are well accepted, because they present no ethical or public concerns and no conflict with the legislation in most countries. The mating strategies can be followed by adaptation to inhibitory compounds as this procedure rendered the hybrid strains in this study slightly more tolerant to ethanol.

Furthermore, a comparison of the parent and hybrid strains could be conducted on molecular level to evaluate transcription and protein expression levels during fermentation in either selective or non-selective conditions. The copy number of the genes responsible for superior characteristics such as fermentation vigour, inhibitor-, ethanol- and osmotolerance between the hybrid strains and the parent strains can be compared. If the copy number of the genes in the hybrid strains is low, the genes can be over expressed and evaluated against the initial hybrid strains to see if the hybrid strains display improved characteristics (consume glucose faster and produce higher yields of ethanol).

5.1 References

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5.2 Appendix:

5.2.1 Mating results of remaining hybrids

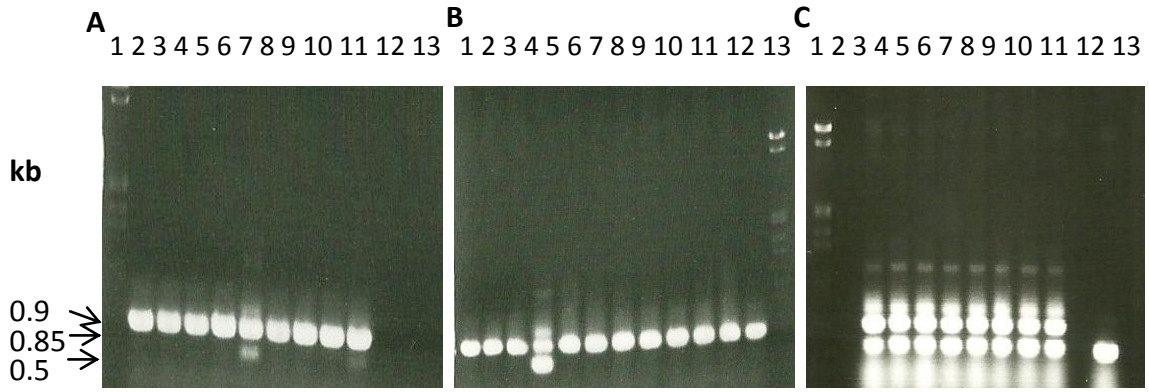

Figure 35: Agarose gel electrophoresis of PCR products obtained for the *MAT***a**/α PCR of the remaining hybrids. (A) Represent the hybrids obtained from mating experiments between the spores obtained from the *S. cerevisiae* YI64 and HR14 strains with lane 6 showing the YH2 hybrid strain and (B) lane 4 showing the YH3 hybrid strain. (C) Represents the rest of the hybrid strains obtained from mating experiments between the spores from the *S. cerevisiae* MF15 and YI2 strains.

5.2.2 ANOVA results for fermentations

5.2.2.1 Parental strains HR14, YI64 and hybrids YH1, YH2 and YH3:

Glucose fermentations

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	6637.0102	<.0001	554.373	0.0007
Strain (Rep)	10	152.6273		44.448	
Period (Days)	5	58998.7495	<.0001	16394.750	<.0001
StrainsxPeriod	20	361.4038	0.2302	55.962	0.0156
Error	50	280.4091		26.233	
Corrected Total	89				

Fermentations in the presence of 25% inhibitor cocktail

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	382.657841	0.4245	0	0
Strain (Rep)	10	360.57275		0	
Period (Days)	4	4628.00952	<.0001	0	0
StrainsxPeriod	16	255.24921	0.617	0	0
Error	40	297.40936		0	
Corrected Total	74				

Fermentations in the presence of 10% ethanol

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	217.821951	0.8858	29.001828	0.2401
Strain (Rep)	10	784.599985		17.719891	
Period (Days)	4	1118.184918	0.0005	1586.443731	<.0001
StrainsxPeriod	16	183.534285	0.4351	23.099214	0.2676
Error	40	175.71486		18.299537	
Corrected Total	74				

Fermentations after adaptation to 10% ethanol

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	3	1007.387764	0.0387	90.075847	0.0463
Strain (Rep)	8	221.855833		21.412992	
Period (Days)	4	317.082122	0.012	1641.617019	<.0001
StrainsxPeriod	12	219.511782	0.0141	46.276337	<.0001
Error	32	83.11778		6.124169	
Corrected Total	59				

Fermentations on triticale straw hydrolysate

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	3	1107.507491	<.0001	178.5657617	0.0001
Strain (Rep)	8	25.41624		6.0213417	
Period (Days)	4	784.060556	<.0001	153.3385692	<.0001
StrainsxPeriod	12	152.689887	<.0001	37.2720769	0.0004
Error	32	11.215457		8.389738	
Corrected Total	59				

5.2.2.2 Parental strains MF15 and YI2 and hybrids MY3, MY5 and MY7:

Glucose fermentations

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	2256.9761	0.0738	233.25823	0.0365
Strain (Rep)	10	758.6862		59.61989	
Period (Days)	5	78200.676	<.0001	13775.33668	<.0001
StrainsxPeriod	20	337.4429	0.0002	33.18627	0.1459
Error	50	99.4945		22.96233	
Corrected Total	89				

Fermentations in the presence of 25% inhibitor cocktail

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	2299.4098	0.0005	341.23472	0.033
Strain (Rep)	10	172.1144		84.12051	
Period (Days)	3	62508.7649	<.0001	8739.85587	<.0001
StrainsxPeriod	12	614.1794	<.0001	175.81502	0.0022
Error	30	72.3313		48.82464	
Corrected Total	59				

Fermentations in the presence of 10% ethanol

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	4	20.9077313	0.8882	10.860941	0.3733
Strain (Rep)	10	76.3423107		9.134665	
Period (Days)	4	75.5651013	0.0519	2374.428755	<.0001
StrainsxPeriod	16	22.8850688	0.6968	3.135997	0.2162
Error	40	29.313327		2.322859	
Corrected Total	74				

Fermentations after adaptation to 10% ethanol

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	3	1499.117819	0.0128	104.345317	0.0646
Strain (Rep)	8	215.457543		28.818658	
Period (Days)	4	703.108236	<.0001	1852.656868	<.0001
StrainsxPeriod	12	235.144651	0.0006	47.693628	0.0029
Error	32	56.72405		14.128358	
Corrected Total	59				

Fermentations on triticale straw hydrolysate

		Glucose		Ethanol	
Source	DF	Mean Square	P-value	Mean Square	P-value
Strains	3	442.337659	<.0001	98.1419444	0.0332
Strain (Rep)	8	5.024245		20.2971033	
Period (Days)	4	1081.090598	<.0001	185.8750308	<.0001
StrainsxPeriod	12	93.017627	0.0005	33.5346264	0.0017
Error	32	21.768783		9.220295	
Corrected Total	59				